The diagnosis and pathophysiology of tuberculous meningitis in Vietnamese adults

Thesis

THE DIAGNOSIS AND PATHOPHYSIOLOGY OF TUBERCULOUS MENINGITIS IN VIETNAMESE ADULTS

Dr Guy Edward Thwaites MA MBBS MRCP

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Wellcome Trust Research Unit
The Hospital for Tropical Diseases
Ho Chi Minh City
Viet Nam

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ProQuest LLC.
789 East Eisenhower Parkway
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ABSTRACT

Approximately 30% of people with tuberculous meningitis (TBM) die despite modern treatment. Survival is dependent upon early treatment but diagnosis is difficult: the clinical features are non-specific, conventional bacteriology is widely regarded as insensitive, and newer diagnostic tests are incompletely evaluated. In addition, the pathogenesis of TBM remains so poorly understood that prospects for new interventions to improve outcome are few.

This thesis examines the diagnosis and pathophysiology of TBM in adults admitted to an infectious disease hospital in Ho Chi Minh City, Vietnam. The aim was to address three questions: what is the best method for distinguishing TBM from other central nervous system disorders, how does disease pathophysiology relate to treatment and clinical outcome, and what other variables influence prognosis?

Three methods for the diagnosis of TBM were studied: clinical, bacteriological and molecular. A diagnostic rule developed from five clinical features predictive of TBM was 86% sensitive and 79% specific when applied prospectively. A bacteriological diagnosis of TBM was confirmed in 107/132 (81%) adults: acid-fast bacilli were seen in 58% and cultured from 71%. Volume of CSF, duration of symptoms, CSF neutrophils, lactate and glucose all predicted bacteriological confirmation. The sensitivity and specificity of CSF Ziehl-Neelsen stain (52% and 100%) was greater than nucleic acid amplification (Gen-Probe Amplified Mycobacterium tuberculosis Direct test) (38% and 99%), although the combined performance of these tests on serial samples detected 83% of cases.

The pathogenesis of TBM was investigated by identifying clinical and molecular markers of poor outcome. Treatment before the onset of coma independently predicted survival, and death was associated with high CSF concentrations of lactate, low numbers of white cells, in particular neutrophils, and low CSF glucose. CSF lactate concentration is a good indicator of disease severity and CSF bacterial load, and neutrophils may have a hitherto unreported protective role.

ACKNOWLEDGEMENTS

The work described in this thesis would not have been possible without the participation of the adults admitted to the Clinical Research Unit (CRU). I am indebted to them and their relatives for their consent to take part in the studies described within this thesis.

There are many people in the CRU who have helped care for these patients and recorded the necessary clinical data. Dr TT H Chau co-coordinated the clinical care of the patients and ensured the study notes were kept accurate and up-to-date. She was responsible for collection of all the clinical data before my arrival in Vietnam in 2000, from which the clinical diagnostic algorithms (**Chapter 3**) were developed. However, I am extremely grateful to all of the Doctors and Nurses of the CRU – without them, this work would not have been possible.

Before I acknowledge those who have helped with the various technical aspects of this research, I would like to thank those who first saw the important questions TBM posed to the CRU and have overseen my efforts to answer them. Five years ago, one wet afternoon in Oxford, Professor Nicholas White suggested my wife and I should work in Vietnam and we are enormously grateful for the opportunity and help he has given us. A similar magnitude of thanks also goes to Dr Jeremy Farrar, my supervisor, who has been a constant source of support, enthusiasm and ideas. I would also like to thank Dr TT Hien for his guidance and shrewd advice on how to conduct clinical research in Vietnam, and his never-ending patience with my attempts to follow it.

The following people have also helped with the research described in this thesis and deserve many thanks. Dr Kasia Strepniewska has been an invaluable source of

statistical advice and together we constructed the diagnostic algorithms presented in **Chapter 3**. Dr Chris Parry and Mr Jim Campbell taught me the techniques of tuberculosis bacteriology and covered the TBM diagnostic service when I was away. Dr Maxine Caws performed the spoligotyping described in **Chapter 8** and the MTD test described in **Chapter 5**. Dr Cameron Simmons supervised the laboratory work described in **Chapter 6** and was an indispensable source of advice and ideas. Professor David Ferguson (University of Oxford, UK) took the electron micrographs (**Chapter 6**). Particular thanks also goes to Jeanne Packer in Oxford, who tirelessly responded to my frequent requests for past publications and posted them to Vietnam. I also thank the Wellcome Trust of Great Britain for funding the work.

Finally, I thank Louise, my wife, not only for correcting and proofreading this thesis, but also for her unstinting support and common-sense advice concerning this research and much, much more besides.

DECLARATION

Other than the assistance outlined in the acknowledgements, the work described in this thesis is my own work and has not been submitted for a degree or other qualification to this or any other university.

The whole thing lasted scarcely two weeks, including the earliest signs that all was not quite well with the child; from the beginnings no one – I believe no one at all – even dreamed of the horror to come....

Kurbis tested the child's eyes, the pupils of which were tiny and showed a tendency to squint. The pulse raced. Muscular contractions developed, and an incipient rigidity of the neck. It was cerebro-spinal meningitis, inflammation of the meninges. The good man pronounced the name with a deprecating movement of the head shoulder wards, probably in the hope that they might not know the almost complete powerlessness of medical science in the face of this onslaught.

Thomas Mann describes the death of a child from tuberculous meningitis before the advent of chemotherapy in his novel *Dr Faustus*, (Mann T, 1947).

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ABBREVIATIONS

AFB	Acid-Fast Bacilli
AI	Albumin Index
ATC	Anti-Tuberculosis Chemotherapy
ATS	American Thoracic Society
BBB	Blood brain barrier
BCG	Bacille Calmette- Guerin
BM	Bacterial Meningitis
BTS	British Thoracic Society
CDC	Centers for Disease Control
CI	Confidence Interval
CNS	Central Nervous System
CRU	Clinical Research Unit
CSF	Cerebrospinal Fluid
CT	Computerised tomography
DI	Diagnostic Index
DNA	Deoxyribonucleic acid
DOT	Directly Observed Therapy
ELISA	Enzyme-linked Immunosorbent Assay
FDA	Food and Drug Administration
GCS	Glasgow Coma Score
HIV	Human Immunodeficiency Virus
HTD	Hospital for Tropical Diseases

ICP	Intra-cranial Pressure
IFN	Interferon
IgGI	IgG Index
IL	Interleukin
IUATLD	International Union Against Tuberculosis and Lung Disease
LJ	Lowenstein-Jensen
MAB	Monoclonal Antibody
MGIT	Mycobacterium Growth Indicator Tube
MIC	Minimum Inhibitory concentrations
MMP	Matrix Metalloproteinase
MRC	Medical Research Council
MRI	Magnetic Resonance Imaging
M.tb	***Mycobacterium tuberculosis***
MTD	Mycobacterium Tuberculosis Direct test
NAA	Nucleic acid amplification
PAS	Para-Amino Salicylic acid
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
PPD	Purified Protein Derivative
ROC	Receiver Operator Characteristic
TBM	Tuberculous Meningitis
TIMP	Tissue Inhibitor of Metalloproteinase
TNF	Tumour Necrosis Factor
TSA	Tuberculosteric acid
UV	Ultra-violet

WHO	World Health Organization
ZN	Ziehl-Neelsen

CHAPTER 1

INTRODUCTION

1.1 Tuberculosis

In the seventeenth century John Bunyan described tuberculosis as, 'The Captain of all these Men of Death'. In the late twentieth century the World Health Organisation (WHO) pronounced it a 'global emergency'. Whether described by poetry or prose, tuberculosis has been the leading global infectious cause of morbidity and mortality for more than 500 years.

In the early twentieth century tuberculosis declined amongst the richer nations. In 1900 the incidence of tuberculosis in the United States was approximately 250 per 100,000, with a death rate greater than 100 per 100,000 (Iseman MD, 2000). By 1973 the incidence had fallen to 15/100,000, with a death rate of less than 2/100,000. These figures fuelled enormous optimism best encapsulated in 1969 by William Stewart, Surgeon General to the United States, when he suggested it was, 'time to close the book on infectious disease.'

Unfortunately, the figures for the rest of the world told a different story. In 1995 the WHO published an overview of the global impact of tuberculosis (Raviglione MC *et al.*, 1995). They estimated that one in three people was infected with the tubercle bacillus, and that in 1990 there were approximately 8 million new cases of active tuberculosis and 2.6 million deaths. The estimated tuberculosis incidence rates by country are presented in **Figure 1.1**.

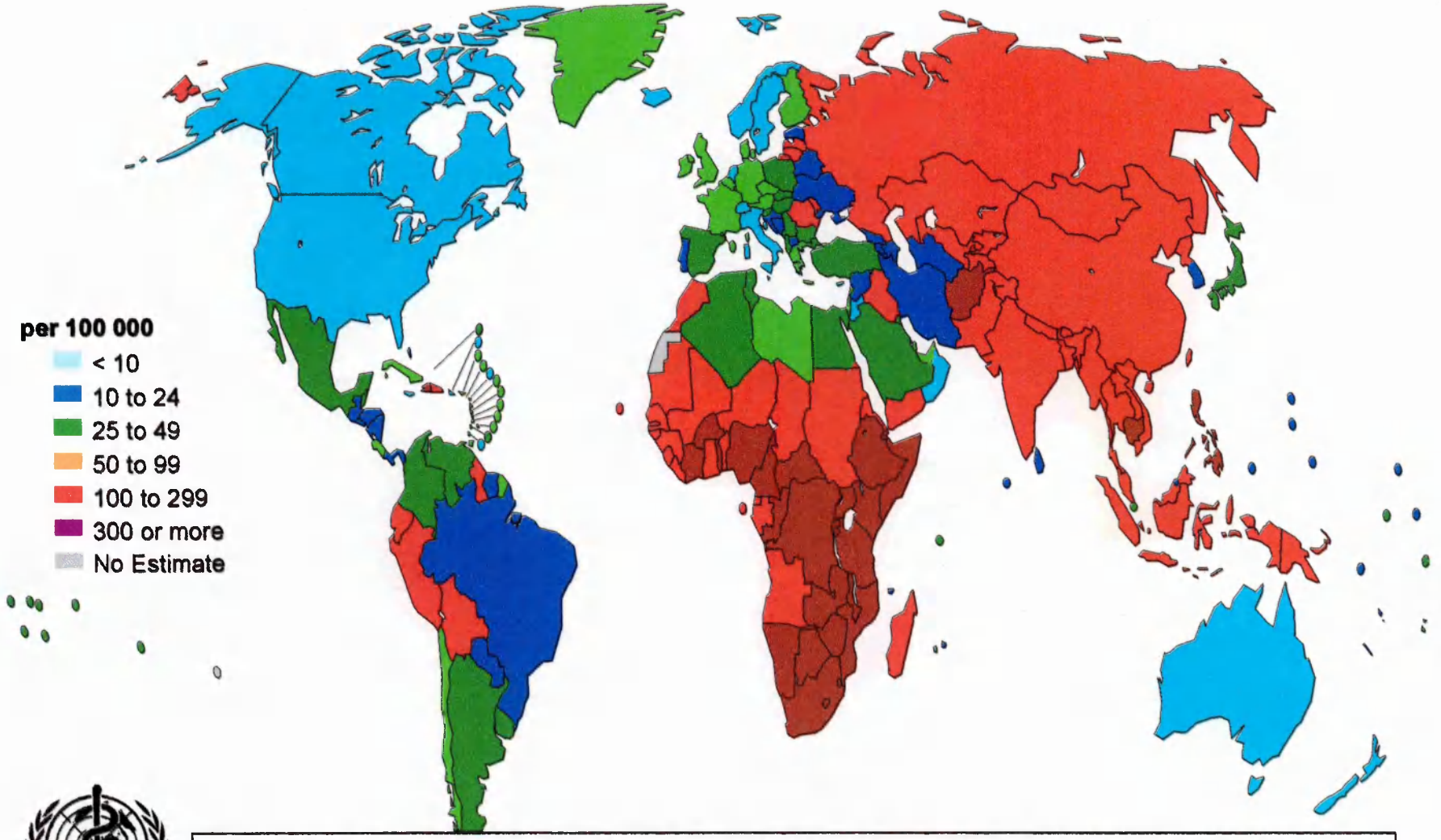


Figure 1.1 Estimated tuberculosis incidence rate worldwide, 2002, according to the WHO (WHO, 2003)

1.1.1 History of tuberculosis

Humans have probably suffered from tuberculosis for more than 5000 years. Examination of Egyptian mummified human remains from around 3400 B.C have revealed dorsal spine deformities consistent with spinal tuberculosis (Cave AJE, 1939), and there are allusions to a group of pulmonary and extra-pulmonary diseases that probably represent tuberculosis in ancient Chinese writings from around 3 000 B.C (Iseman MD, 2000). But it was not until the time of Hippocrates (circa 460-375 B.C), and the flowering of Greek medicine, that tuberculosis first received systematic study. The Greeks called it *Phthisis*, which means 'I am wasting', and remains an appropriate clinical description of tuberculosis to the present day. But despite increasing clinical recognition, the Greek understanding of the disease remained rudimentary. Unfortunately, Aristotle's (354-322 B.C) prescient observation fell unheeded:

> *Why when one comes near consumptives does one contract their disease, while one does not contract dropsy, or apoplexy...? With consumption, the reason is that the breath is bad and heavy.... One takes the disease because there is in the air something disease-producing.*

It was to be another 1860 years until Girolamus Frascatorius (1483-1553) re-examined Aristotle's observations. His book De Contagioni (1546) concluded that there were three major routes of infection transmission: spread by direct contact; spread by an intermediary, or fomite; and spread over a distance involving tiny infectious particles which he called 'seminaria'. The contagious theory of disease was

born, but remained highly controversial for a further 300 years and unaccepted by many physicians caring for patients with phthisis during this period.

The Age of Enlightenment brought new rigor to the study of phthisis. At the beginning of the seventeenth century, the English philosopher Francis Bacon formulated the 'scientific method': the generation of testable hypotheses from observable facts. For physicians this meant careful clinical observation of the living, and autopsy of the deceased. The post-mortem observations of Franciscus de la Boe, Professor of Anatomical Medicine at Leiden, were the first to describe the profusion of nodules within the organs of phthisical patients, and refer to them as 'tubercles'. Then, in 1821, the French physician Laennec recognised that all the diverse clinical forms of 'tubercle' were a single disorder (Laennec RTH, 1821), although his thesis only gained complete acceptance with the discovery of the causative agent of 'tuberculosis'.

It took 217 days for Robert Koch to make that discovery (Brock TD, 1988). He began his first experiments on August 18th, 1881; reported his findings to the Berlin Physiological Society on March 24th, 1882; and published the paper three weeks later (Koch R, 1882). During this period Koch stained and cultured the bacillus for the first time, demonstrated its presence in tuberculous tissue, and inoculated guinea pigs with the culture to reproduce the disease. He concluded:

> *All these facts taken together lead to the conclusion that the bacilli which are present in the tuberculous substances not only accompany the tuberculosis process, but are the cause of it. In the bacillus we have, therefore, the actual tubercle virus. (Koch R, 1882)*

1.1.2 Tuberculosis nomenclature and bacteriology

Koch's tubercle bacilli belongs to the genus *Mycobacterium* (meaning 'fungus-bacterium'), members of which share the property of 'acid fastness', the characteristic ability to resist decolourisation in the presence of a weak mineral acid after staining with an arylmethane dye (Collins CH *et al.*, 1997). In 1898 Theobold Smith divided the tubercle bacilli into human and bovine varieties (Smith T, 1898): *Mycobacterium tuberculosis* and *Mycobacterium bovis*. To these have been added *Mycobacterium microti* (Wells AQ, 1946), *Mycobacterium africanum*, the vaccine strain Bacille Calmette-Guerin (BCG) (Skerman VDB *et al.*, 1980), and more recently *Mycobacterium canetti* (van Soolingen D *et al.*, 1997). Together they are referred to as '*Mycobacterium tuberculosis* complex', and only members of this group can cause human 'tuberculosis', although *Mycobacterium microti*, BCG, and *Canetti* rarely cause disease. Other members of the genus *Mycobacterium* can cause disease in humans, particularly in immune compromised individuals, but these diseases are not labelled as 'tuberculosis'.

This thesis is concerned only with '*Mycobacterium tuberculosis* complex' infection, although for ease and consistency the agent will simply be referred to as *Mycobacterium tuberculosis* (*M.tb*).

1.1.3 Tuberculosis infection and transmission

As with many infectious diseases, the number of people suffering from tuberculosis disease is a fraction of the total number infected. Estimates of the number infected have conventionally been calculated by intra-dermal reactivity to a purified protein

derivative (PPD) of *M.tb* (ATS, 1990). Reactivity is taken to represent exposure with infection: one in three people are believed infected, and it is estimated that one in ten of these will develop active disease (Raviglione MC *et al.*, 1995). Active disease is broadly divided into three categories: sputum smear-positive pulmonary disease; sputum-smear negative pulmonary disease; and extra-pulmonary disease. Smear-positive disease is defined by demonstration of acid-fast bacilli (AFB) within a clinical specimen by light microscopy. From an epidemiological perspective, individuals with sputum smear-positive disease are the most important, as they represent infectious cases and are responsible for the majority of tuberculosis transmission (Grzybowski S *et al.*, 1975). The WHO, and International Union against Tuberculosis and Lung Disease (IUATLD), hold the detection and treatment of sputum smear-positive cases to be a fundamental tenet of tuberculosis control (WHO, 1997) (Reider HL, 2002).

Much of the current understanding of the airborne transmission of tuberculosis comes from a series of elegant experiments performed by Riley between 1956 and 1961 (Riley RL *et al.*, 1961). Riley designed a ward for patients with tuberculosis in which the air from each room was ducted into cages containing guinea pigs (which are naturally highly susceptible to *M.tb* infection) (Riley RL *et al.*, 1962). The air leaving each room was split into equal columns, each being delivered to a different group of animals, with one column being irradiated with ultra-violet (UV) light. Monthly PPD skin testing of all animals identified those newly infected, and those found to be reactive were killed and dissected. There were no infections amongst the guinea pigs

breathing UV irradiated air, but amongst the rest the average time to infection was 10 days. Untreated patients and one adult with tuberculous laryngitis were responsible for the majority of infections. The study findings validate the theory of droplet-generated airborne transmission of tuberculosis, and supported the premise that the likelihood of transmission increased with the concentration of airborne infectious particles and the volume of air inhaled.

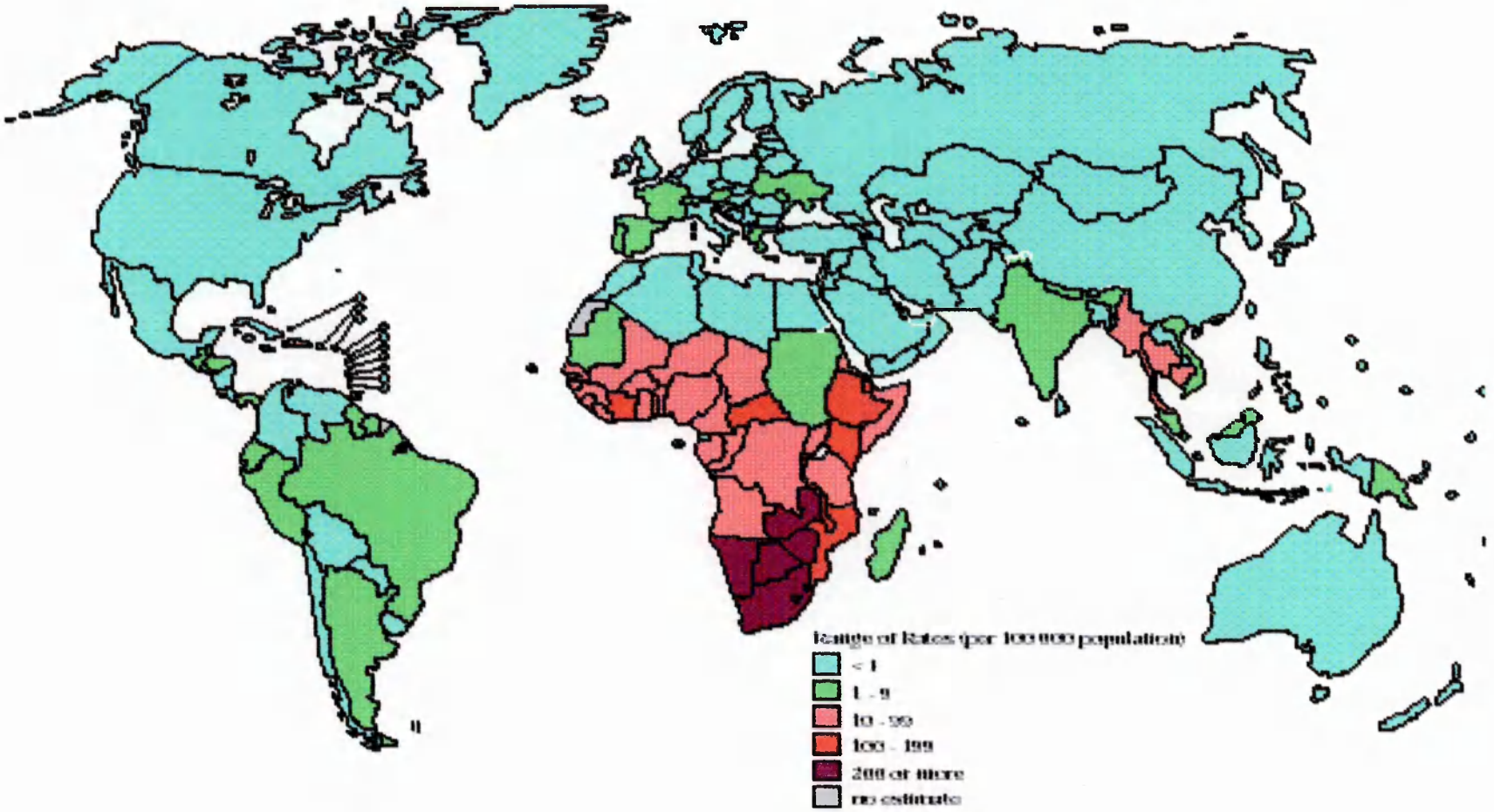
Tuberculosis transmission by other routes can occur, but is rare. Infection with *Mycobacterium bovis* (a member of the *Mycobacterium tuberculosis* complex) through ingestion of contaminated cows milk used to be a significant global cause of tuberculosis but pasteurisation has largely removed this risk, alongside case detection with PPD skin tests and slaughter of infected animals. However, in regions with limited access to these preventative measures, this route of transmission remains a major public health problem.

1.1.4 Risk factors for tuberculosis

There are two broad categories of risk for tuberculosis: environmental, and biological. Environmental risk factors determine the intensity and duration of exposure of an individual to other individuals with infectious tuberculosis. Prominent amongst this group are prisons, nursing homes, homeless shelters, and hospitals. High transmission rates and well-documented outbreaks have been reported in all of these institutions (Stead WW, 1978; Stead WW, 1981; Schieffelbein CW, Jr. *et al.*, 1988; Sepkowitz KA, 1994). However, sustained physical proximity is not the only reason these

environments present special risk. Their populations frequently possess numerous biological reasons for increased susceptibility to tuberculosis. Alcohol dependence, intra-venous drug use, and human immunodeficiency virus (HIV) infection are common biological risk factors shared by the homeless and those in prisons (Iseman MD, 2000). Other important biological risk factors, often shared by those in nursing homes or hospitals, are diabetes mellitus (Kim SJ *et al.*, 1995), corticosteroid therapy (Sahn SA *et al.*, 1976), gastrectomy (Snider DE, Jr., 1985), end-stage renal disease (Andrew OT *et al.*, 1980), silicosis (Snider DE, Jr., 1978), and malnutrition (ATS, 1994). Most recently, tobacco smoking has been shown to be a major risk factor for disease and death from pulmonary tuberculosis (Gajalakshmi V *et al.*, 2003). Many of these factors are believed to alter the immune response to infection with *M.tb*, and compromise the individual's ability to resolve new infection or control reactivation of latent bacilli. The clearest example is HIV infection, which alters the risk of reactivation from 1 in 10 to 1 in 3 (Selwyn PA *et al.*, 1989), and increases the likelihood of progressive disease after new infection with *M.tb* (Di Perri G *et al.*, 1989). The HIV epidemic has had, and will continue to have, an enormous impact on the global prevalence of tuberculosis. WHO estimate that there are 40 million people currently infected with HIV of whom approximately 13 million are co-infected with tuberculosis (WHO, 2002). Estimated numbers of HIV-infected tuberculosis cases per 100,000 population in 2000 are shown in **Figure 1.2**. Conservative estimates, which take no account of rates of new infection, suggest that 4 million will reactivate and require treatment for active tuberculosis. Consequently, HIV infection dwarfs all other risk factors for tuberculosis.

Figure 1.2 Estimated numbers of HIV-infected TB cases per 100,000 population in 2000 (WHO, 2002)



1.1.5 Clinical Tuberculosis

Tuberculosis can affect any organ of the body, but the first site of infection is nearly always the lung. In settings of high tuberculosis prevalence this 'primary infection' usually occurs in childhood and is asymptomatic in more than half (Starke JR *et al.*, 1989). In the rest, the symptoms of cough and fever predominate (Pineda PR *et al.*, 1993). In most symptomatic cases the chest X-ray reveals hilar, mediastinal, or paratracheal lymphadenopathy (**Figure 1.3**), and there is evidence of lung parenchymal involvement in around 75% (Pineda PR *et al.*, 1993). The majority of primary infections are uncomplicated, but on rare occasions progressive primary tuberculosis develops: lung parenchymal involvement increases in the form of an

Figure 1.3 Primary tuberculosis with hilar lymphadenopathy (arrow) (Hospital for Tropical Diseases [HTD])

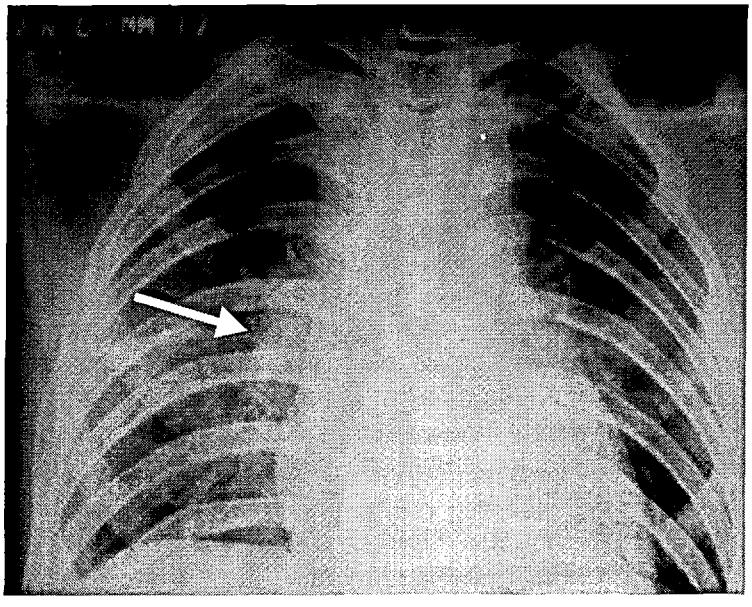
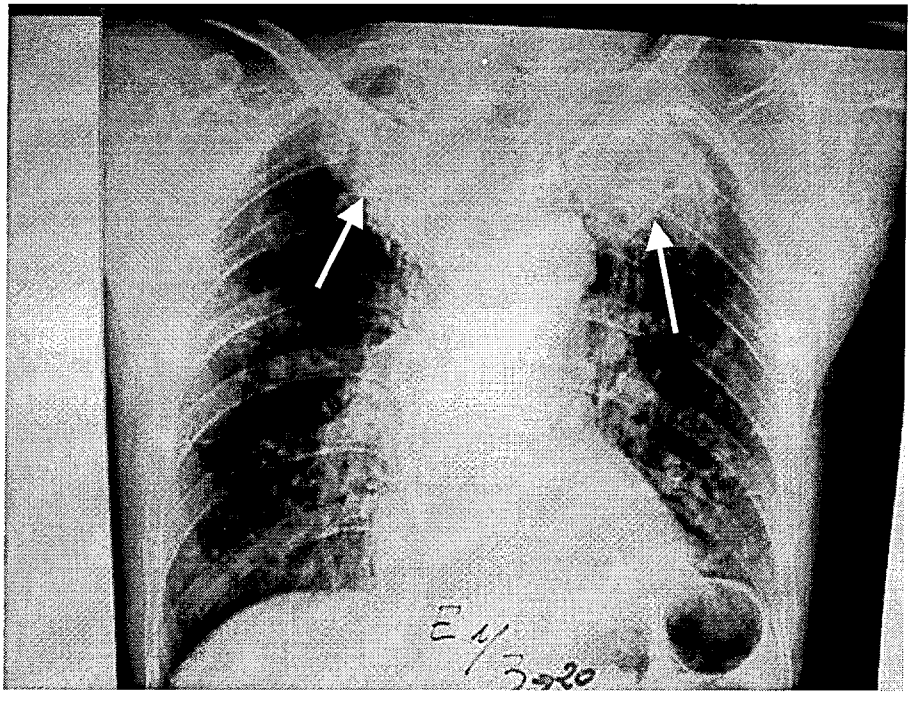


Figure 1.4 Typical Chest X-ray appearances of pulmonary tuberculosis: bilateral apical shadowing and cavities *(arrows) (HTD)*



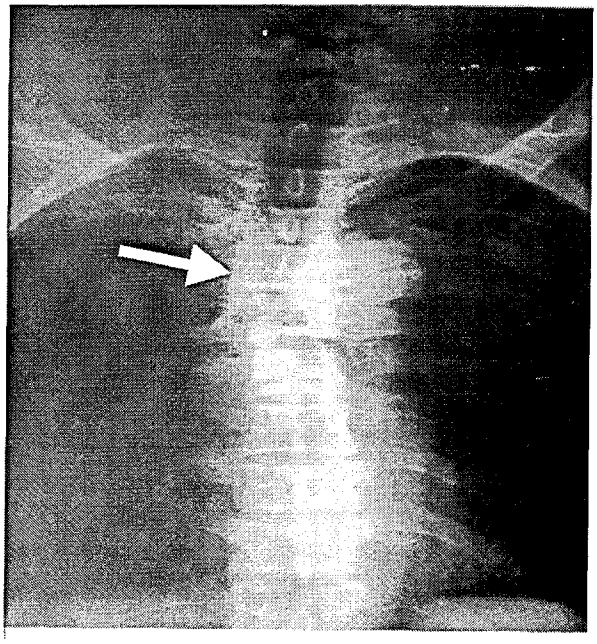
intense and localised pneumonia, sometimes leading to the formation of cavities and often with haematogenous dissemination. The prognosis of this type of disease is poor.

The traditional distinction of primary from post-primary tuberculosis depends upon whether disease occurred before or after 5 years from the initial infection (Iseman MD, 2000). This arbitrary definition is only useful if all post-primary disease is considered to be consequent to endogenous reactivation of latent bacilli. There are increasing data to suggest this assumption is incorrect (Fine PE *et al.*, 1999). Recent studies using DNA fingerprinting techniques have shown that substantial numbers of adult post-primary pulmonary disease is due to re-infection with a different strain of

M.tb (van Rie A *et al.*, 1999; Chaves F *et al.*, 1999). However, whilst re-infections may be more important than previously thought, the majority of disease in low prevalence settings is due to reactivation (Fine PE *et al.*, 1999).

Pulmonary tuberculosis is the commonest clinical phenotype, accounting for approximately 80% of all forms of tuberculosis (Iseman MD, 2000). Patients usually present with the symptoms of cough, fever, weight loss, and blood is often reported in the sputum (haemoptysis). Pulmonary tuberculosis is usually a slowly progressive condition: patients present with many days, if not weeks, of gradually worsening symptoms. Rapidly progressive disease does occur, particularly in some racial groups believed to be more susceptible to the disease (Stead WW, 1992). Physical examination is often unremarkable: the wasted, pthisical characteristics noted by the Greeks may not be present in early disease; chest examination is frequently normal (it is harder to elicit physical signs from the affected lung apices than the middle and lower lobes); and extra-pulmonary signs may be few. Fever is not universal, and in the elderly, in particular, may be absent (Korzeniewska-Kosela M *et al.*, 1994). Despite limited physical signs, X-ray of the chest will reveal the characteristics and extent of the disease (**Figure 1.4**). For reasons that remain obscure, pulmonary tuberculosis most frequently involves the lung apices (Balasubramanian V *et al.*, 1994). Complications of the disease include the formation of pulmonary cavities, sometimes with massive haemoptysis as a result of arterial invasion, pneumothorax with broncho-pleural fistulae, and contiguous spread to the mediastinum (the pericardium, for example), pleura and vertebral column (**Figure 1.5**).

Figure 1.5. Tuberculous destruction of the thoracic vertebrae (arrow) **(Potts disease) arising from secondary spread from the lung** (HTD)



The advent of anti-tuberculosis chemotherapy (ATC) dramatically reduced the incidence of these complications, although they are being seen again in those with multi-drug resistant tuberculosis (Tahaoglu K *et al.*, 2001).

Haematogenous dissemination of the bacilli can occur at any time during infection with *M.tb,* and can result in infection of any organ (see **Figures 1.6** and **1.7**). Commonly affected are the brain, the kidneys, the bones and the cervical lymph nodes that drain the pulmonary vessels. In the United States in 2001, there were 15,989 cases of tuberculosis reported to the Centre for Disease Control, Atlanta (CDC): 12,768 (80%) were pulmonary, and 3,212 (20%) were extra-pulmonary (CDC, 2001).

Figure 1.6 Disseminated extra-pulmonary tuberculosis: bilateral fusiform psoas abscesses (thick arrows) **with vertebral osteomyelitis** (thin arrow) **by MRI** (HTD)

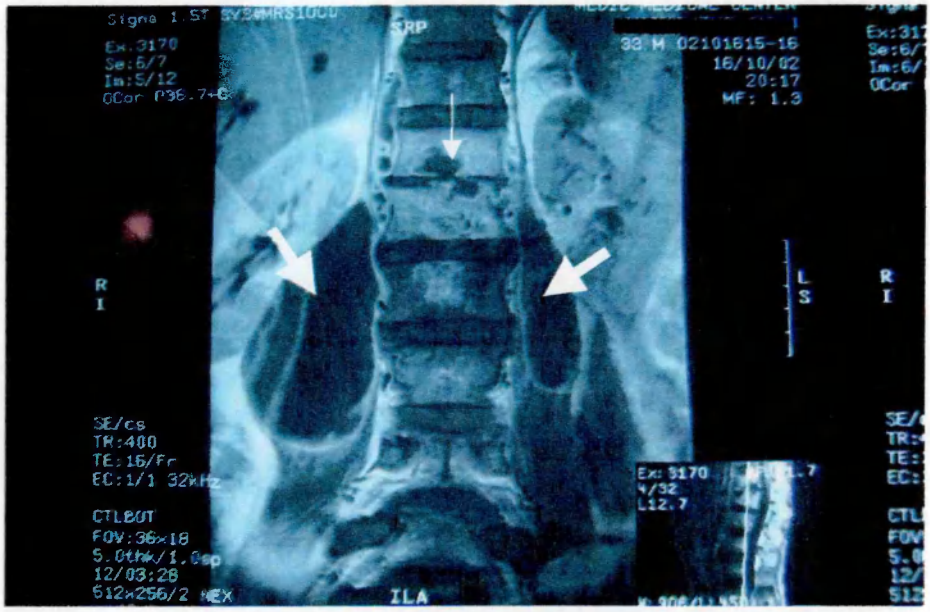
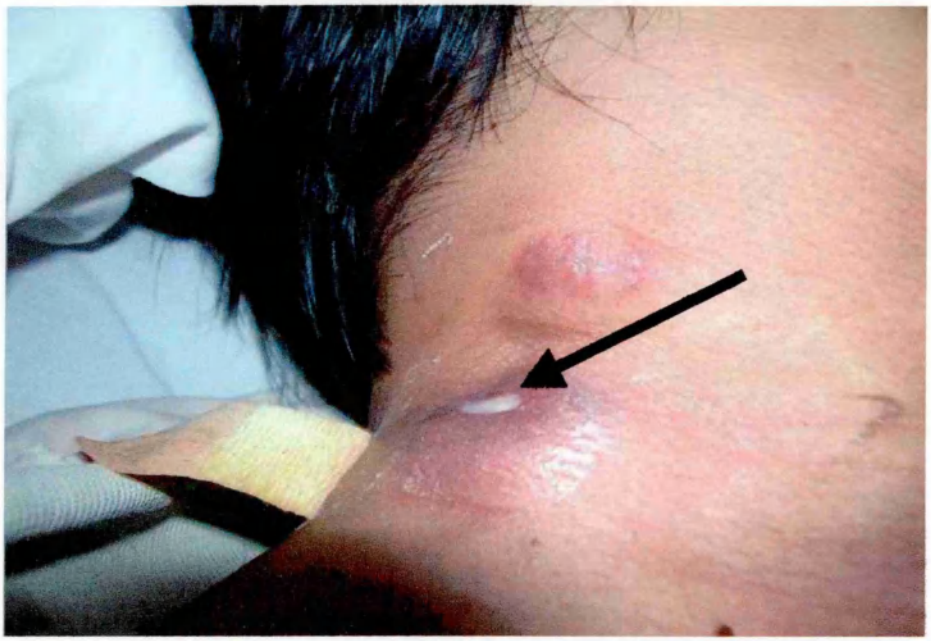


Figure 1.7 Discharging tuberculous lymph node (arrow), **or scrofula, in an HIV infected adult with tuberculous meningitis** (HTD)



The extra-pulmonary cases reported were pleural (19%), lymph node (42%), bone or joint (10%), genito-urinary tract (6%), meningeal (6%), peritoneal (4%), and other sites (13%).

The proportion of extra-pulmonary cases has risen over the last 40 years in the United States and other wealthy countries for reasons that are poorly defined. HIV infection increases the risk of disseminated disease and is an important component of this change. Also important are the changes in the demographic profile of those with tuberculosis: in affluent countries, such as the United States, the burden of disease now falls amongst racial and ethnic minorities and recent immigrants.

The clinical manifestations of extra-pulmonary tuberculosis are as diverse as the organs it can affect, and it is not within the scope of this thesis to review them all. However, the non-specific clinical presentations, the difficulty in obtaining adequate diagnostic samples, and the paucity of bacilli causing disease, all serve to make the diagnosis difficult. Tuberculous meningitis, the focus of this thesis, provides an excellent example of all of these problems.

1.1.6 Diagnosis of tuberculosis

For most of the world the diagnostic methods for tuberculosis have not changed for 120 years. Only recently have efforts been made to develop new diagnostic assays, some of which are available in the laboratories of wealthy countries. Fortunately, the diagnostic method developed by Franz Ziehl (1857-1926) and Friedrich Neelsen (1854-1894) in 1882 has sufficient diagnostic sensitivity and specificity to detect

those with the most infectious forms of the disease, and therefore forms the basis of global tuberculosis control programmes. The Ziehl-Neelsen (ZN) stain depends upon the 'acid-alcohol fast' biological properties of *M.tb*. Paul Ehrlich first elucidated these properties in 1882 (Ehrlich P, 1882), and they were developed into a test by Ziehl and Neelsen. Neelsen introduced the use of the dye basic fuchsin and found that tubercle bacilli were identifiable from nearly all other species of bacteria by their ability to resist decolouration with weakly acidified alcohol (Neelsen F, 1883). He found the red stained bacilli were easier to visualise with the background counter-stained with methylene blue (**Figure 1.8**). Small variations on this method exist, but the principles remain unchanged today.

The demonstration of AFB in the sputum of a patient suspected of tuberculosis is the primary diagnostic method for much of the world. However, there are some important limitations to this technique. First, the sensitivity of a direct sputum smear is between 50-60% (Collins CH *et al.*, 1997). Examining at least 3 specimens, and concentrating the specimen by centrifugation after the addition of a mucolytic agent can increase the sensitivity. Second, it is impossible to tell the species of mycobacteria by microscopy. Other mycobacteria (*Mycobacterium kansasii*, for example) can cause pulmonary disease, or can contaminate respiratory specimens without causing disease. Only culture of the mycobacteria will allow a full identification and confirm the diagnosis of tuberculosis. More recently, fluorescence microscopy using the auromine-rhodamine stain has improved the speed and ease of microscopy. The staining principles are exactly the same, except the auromine-rhodamine stained bacilli fluoresce when excited with UV light. The bacilli are easier to see than by ZN

Figure 1.8 Acid-fast bacilli in cerebrospinal fluid (arrow) (HTD)

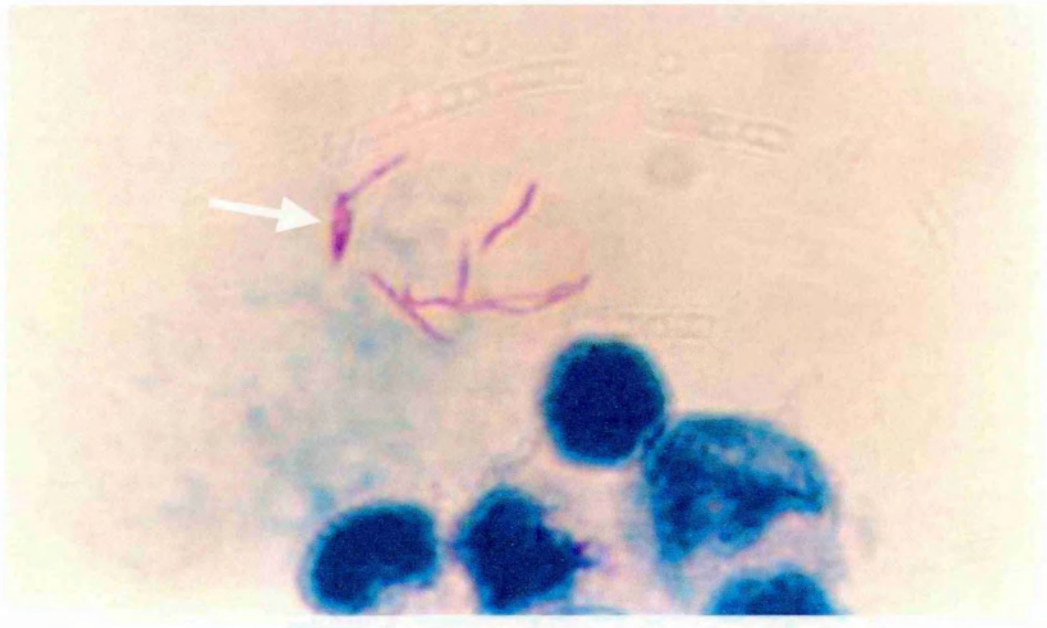


Figure 1.9 Culture of Mycobacterium tuberculosis on Lowenstein-Jensen media (HTD)

stain, and slides can be quickly and effectively screened under low magnification. However, when few bacilli are present (in cerebrospinal fluid, for example), fluorescing debris can be mistaken for bacilli, and a confirmatory ZN stain should always be performed.

Confirmation of infection with *M.tb* can only be made by culture, but as Robert Koch found, *M.tb* does not grow well on conventional media. Koch eventually found the bacilli grew well on slopes of coagulated blood serum (Brock TD, 1988), and subsequently many different culture media have been devised. The solid media currently used are egg-based (Lowenstein-Jensen, **Figure 1.9**) and agar based (Middlebrook's 7H10 or 7H11). Liquid media (Middlebrook's 7H9, or Kirchners media) are less selective but especially useful when culturing specimens other than sputum, when the chances of contamination with other bacterial species are low.

Simple growth characteristics will allow the preliminary identification of *M.tb*. The early bacteriologists recognised that cultivable mycobacteria could be divided into 'rapid growers' (growth on egg-media within 5 days) and 'slow growers'. *M.tb*, and most other pathogenic mycobacteria are 'slow growers' – they often take between 4 and 8 weeks to produce significant colonies on egg-based solid media. *M.tb* grows best at 37^0C, is non-pigmented, and demonstrates characteristic 'cords' of bacilli when stained by ZN, but the formal identification rests on an array of confirmatory tests, or more recently by nucleic acid probes (Collins CH *et al.*, 1997). It is not within the scope of this thesis to review these methods, but they are time-consuming and difficult, and are usually only performed by experienced technicians in reference

laboratories. For these reasons, in many settings the culture and identification of *M.tb* remains impossible, and diagnosis rests upon the result of the ZN stain.

The lack of diagnostic sensitivity and specificity of conventional diagnostic methods, and the time required to culture and identify the organism, has led to the development of more rapid, nucleic acid-based techniques. It is now possible to determine whether a specimen contains *M.tb* nucleic acid and is likely to be resistant to rifampicin within a few hours (Watterson SA *et al.*, 2000). However, despite the promise of these methods, their performance and role in clinical decision-making remains unclear (Pfyffer GE, 1999).

The amplification and detection of regions of *M.tb*-specific nucleic acid from clinical specimens is an attractive diagnostic prospect. To date, the Food and Drug Administration (FDA) of the United States have cleared two nucleic acid amplification assays for the direct detection of *M.tb* in respiratory samples: the Amplified Mycobacterium tuberculosis Direct Test (MTD) (Gen-Probe, Inc, San Diego, California) and the AMPLICOR Mycobacterium tuberculosis test (Roche Diagnostics, Inc, Indianapolis, Ind). The performance of these assays was good when testing AFB smear-positive specimens (sensitivity 95-96%, specificity 100%), but less satisfactory when AFB smear-negative sputum was tested (sensitivity 48-53%, specificity 96-99%) (Woods GL, 2001). As a consequence, the FDA limited the licence of these test to smear-positive specimens, and attempts were made to improve the performance of the assays in the smear-negative group. Gen-Probe modified the MTD to enhance sensitivity, and decrease the time-to-result. These efforts were rewarded in September 1999 when the FDA extended the licence of the improved

MTD to include all respiratory samples, regardless of smear result. It remains the only test with a licence extending to all respiratory specimens. To date, the published data suggest the MTD performs well for the diagnosis of pulmonary tuberculosis, regardless of AFB smear result, provided there is a moderate to high suspicion of tuberculosis (Gamboa F *et al.*, 1998; Bergmann JS *et al.*, 1999). These data suggest the sensitivity, specificity, and the positive and negative predictive values of the new MTD are approaching 100% for smear-positive respiratory specimens, and are 83%, 99%, 71% and 99% respectively for smear-negative samples.

These assays are not licensed for use on extra-pulmonary samples, and there are limited data on their diagnostic performance with these types of specimens. Low concentrations of bacilli, small volumes of sample, and assay inhibitors, all contribute to low diagnostic sensitivity (Pfyffer GE, 1999). Attempts to increase diagnostic sensitivity have lead to unacceptable falls in specificity, and the role of the nucleic acid amplification techniques for the diagnosis of extra-pulmonary tuberculosis remains uncertain. These methods will be discussed in more detail below with respect to the diagnosis of TBM.

In summary, the currently available molecular diagnostic methods increase diagnostic speed, but they do not replace AFB smear and culture. On smear-positive specimens they have been shown to be highly reliable in confirming infection with *M.tb.* However, positive results on smear-negative specimens must be interpreted with caution, and treatment decisions should be based on the complete clinical picture.

1.1.7 Treatment of tuberculosis

There was no effective treatment for tuberculosis until 1944. The Greeks and the Romans advocated fresh air and rest: factors they believed could redress the imbalance of the humors causing the disease. Two thousand years later, belief in the therapeutic effect of clean air remained, and led to the development of the tuberculosis sanatoria. Hermann Brehmer is credited with opening the first European 'Kurhaus' for tubercular patients in 1859, inspiring Edward Trudeau to develop a similar institution in the United States at Saranac Lake in 1875. By 1942 there were nearly 100,000 sanatorium beds in the United States and similar numbers in European Alps, providing fashionable retreats for the tubercular middle and upper classes. They inspired some (notably Thomas Mann, who wrote The Magic Mountain in 1924 after his experiences in a sanatorium in Davos, Switzerland), but cured no one. However, institutionalising tuberculosis allowed physicians to experiment with alternative treatments on large numbers of individuals. Some of the methods developed were at best misguided and at worst dangerous and deforming. Pulmonary collapse therapy, either by pneumothorax, phrenic crush, or pneumoperitoneum, led the way, regardless of the lack of evidence for it working.

The chemotherapeutic era began with the inspiration from a soil microbiologist from the Ukraine and a Biochemist from Sweden. Selman Waksman was born in the Ukraine and immigrated to the United States in 1910. By the 1930's he was studying the mechanisms of action of fungal enzymes found in the soil and driven by the possibility of a soil microbe that could kill the tubercle bacillus. In 1943 Waksman's laboratory discovered that *Streptomyces griseus* produced a substance that inhibited

the growth of a wide range of bacteria, including *M.tb*. They named the substance streptomycin.

Jorgen Lehmann, a Dane working in Sweden, approached the same problem from a different perspective. In 1941 a single page report appeared in the journal Science that showed salicylate greatly increased the oxygen uptake of *M.tb* (Bernheim F, 1941). Lehmann reasoned that alteration of the salicylate molecule might inhibit *M.tb* metabolism, and believed the para-amino salt of salicylate was most likely to work. In 1944, despite difficulties with production, Lehmann showed that para-amino salicylic acid (PAS) inhibited growth of *M.tb* in vitro, and was capable of reducing disease in both guinea pigs and humans.

The assessment of these two drugs for the treatment of tuberculosis was a defining moment in medicine. The need for a rapid, authoritative, unbiased assessment was recognised as paramount. The methodology chosen – the random assignment of control and experimental treatment regimens – would become the cornerstone of clinical research. The British Medical Research Council (MRC) performed the first of such trials, comparing streptomycin with bed-rest for acute progressive bilateral pulmonary tuberculosis (MRC, 1948a). This study showed that over 6 months streptomycin reduced mortality and improved bacteriological and radiological cure rates. However, resistance to streptomycin developed in 35/41 patients and after 5 years of follow-up the deaths in the streptomycin group (53%) were only slightly less than in controls (63%). Urgent strategies were required to overcome the problem of resistance and in 1948 the MRC started a trial comparing streptomycin alone, PAS

alone, and the two in combination. It demonstrated unequivocally that combined therapy reduced the risk of acquisition of resistance (Fox W *et al.*, 1956).

Consistent and complete cures only became reality in 1952 with the addition of isoniazid to these two drugs. Although the curative power of this three-drug regimen was recognised quickly by some (Crofton J, 1958), it took some years to become universally accepted. There was a reasonable basis for scepticism: data from randomised trials were slow to appear and conclusive evidence of the efficacy of all three drugs in combination did not become available until 1964 (IUATLD, 1964).

At least 12 months of treatment were required for sustained cure with these regimens. The advent of ethambutol led to its exchange with PAS (which caused frequent side effects), and a better tolerated regimen (Doster B *et al.*, 1973), but reductions in treatment length only became possible with the addition of rifampcin. Rifampicin-containing regimens were tested in three different durations of chemotherapy: six, nine, and twelve months (Brouet G *et al*, 1977). The trial demonstrated that a sustained cure was possible with 9-months of treatment and the term 'short-course chemotherapy' was invented (Fox W, 1981).

A series of trials performed by the MRC in East and Central Africa defined the limits of 'short course chemotherapy', and demonstrated that complete cure could be achieved with 6-months (Fox W *et al.*, 1999). They discovered that pyrazinamide (a drug that was discarded initially due to fears of hepatic toxicity), in combination with rifampicin and isoniazid, showed powerful sterilising activity with rapid conversion to smear-negative sputum. After further trials in Hong Kong, Singapore, Madras and

Algeria, it was shown that the best results were achieved using an 'intensive' phase of 2-months rifampicin, isoniazid and pyrazinamide, followed by a 'continuation' phase of 4-months rifampicin and isoniazid. Thioacetazone or ethambutol could replace rifampicin in the continuation phase, but treatment should be extended by 2 months. The mechanisms of action of the first-line drugs, and ways in which *M.tb* can become resistant to their effects, are presented in **Table 1.1**.

By the late 1970's the best drug combinations and the duration of treatment required had been worked out. Then, in 1986, the tuberculosis trials units of the MRC closed. In 40 years they had delineated all of the measures necessary for successful tuberculosis control, the optimal drug regimens and the importance of directly observed therapy (DOT). Two subsequent events suggest the closure of these units was premature. The first was predictable: the increasing prevalence of drug resistance and resulting treatment failures. The second, the arrival of HIV, was not predictable, although by 1986 there was already clear evidence that tuberculosis and HIV had a special relationship (Raymond CA, 1986)).

Patients with multi-drug resistant tuberculosis (defined as resistance to at least rifampicin and isoniazid) are considered incurable with conventional regimens, and are left untreated in many countries (Farmer P, 2001). Other drugs exist, but to date there have been no randomised controlled clinical trials to evaluate these regimens. HIV threatens all tuberculosis control programmes and presents special problems in clinical management (Small PM *et al.*, 2001). Adverse drug events are more common and standard regimens may be less efficacious. In particular, regimens that do not contain rifampicin have shown a high frequency of failure and relapse

Table 1.1. Mechanisms of action, and modes of resistance, for the first-line anti-tuberculosis drugs (Iseman MD, 2000; Kucers A *et al.*, 1997)

Drug	**Mechanism of action**	**Mechanism of resistance**	**Mutation locus** (prevalence among resistant strains)
Streptomycin	Inhibition of protein synthesis by binding to 30S subunit of *M.tb* ribosomes	i) Mutations of 30S subunit binding site ii) Possible changes in cell permeability to drug	*rpsL* (ribosomal protein subunit 12) (60%) *rcs* (16S ribosomal RNA) (25%)
Ethambutol	Inhibits biosynthesis of *M.tb* cell wall, in particular arabinogalactan (AG) + lipoarabinomannan (LAM)	Mutation in gene encoding arabinosyl transferase causing increased AG+LAM	*embAB* (50%)
Isoniazid	Uncertain. Prodrug activated by *M.tb* catalase peroxidase (*KatG*) inhibits mycolic acid synthesis	Uncertain. Multiple possible mechanisms and loci.	*KatG* (50%) *InhA* (25%) *ahpC* (15%) *kasA* (unknown)
Rifampicin	Inhibits RNA polymerase preventing mRNA production	RNA polymerase subunit B mutation prevent drug binding	*rpoB* (98%)
Pyrazinamide	Unknown. *M.tb* pyrazinamidase converts to active pyazinoic acid	Unknown.	*pncA* (unknown)

(Perriens JH *et al.*, 1991). Antiretroviral therapy introduces new problems when used in combination with ATC: protease inhibitors and the non-nucleoside reverse transcription inhibitors interact with the rifamycins (CDC, 1998), and immune reconstitution can cause paradoxical clinical worsening of tuberculosis (Narita M *et al.*, 1998). The best clinical management of these problems is uncertain, particularly in poorer countries. The relative lack of importance of these issues to wealthy countries and the closure of institutions such as the MRC Tuberculosis Unit previously designed to address them, has reduced the political will to solve them. Moreover, the skills required to perform clinical trials in patients with tuberculosis have been neglected and have to be re-learnt. For all of these reasons, the treatment of tuberculosis remains a formidable challenge.

1.2 Tuberculous Meningitis

Tuberculous meningitis (TBM) is caused by infection of the meninges and parenchyma of the brain or spinal cord with *M.tb*. TBM was invariably fatal before the advent of ATC. The post-mortem observations of Green, published in the Lancet in 1836 (Green PH, 1836), were the first to describe the distinct pathological features of the infection and set it apart from the other recognised causes of 'acute hydrocephalus'. The challenge for the physician then lay in distinguishing the disease before death, and delivering the grave prognosis.

The ability to diagnose TBM became a priority in 1948 after streptomycin was found to reduce mortality by one third (MRC, 1948b). Over the next 5 years progress was rapid: the addition of para-aminosalicylic acid to streptomycin reduced mortality to

30% and the addition of isoniazid to both of these compounds lowered the mortality to around 20% (Lorber J, 1960). However, the mortality from TBM has altered little since the introduction of isoniazid in 1952. The decline of tuberculosis in the developed world over this period lessened the search for new drugs and diagnostic methods. As a consequence, in 2003, the most widely available rapid diagnostic method for TBM remains the stain developed by Ziehl and Neelsen 120 years ago and the anti-tuberculosis drug regimen has not changed since the discovery of rifampicin and pyrazinamide more than 30 years ago.

1.2.1 Epidemiology of tuberculous meningitis

Prior to the arrival of HIV the most important determinant for the development of TBM was age. In populations with high tuberculosis prevalence the peak age of incidence is from 0 - 4 years (Farer LS *et al.*, 1979), and there is close correlation between the incidence of TBM in this age group and the population annual average risk of *M.tb* infection. The childhood incidence of TBM probably represents 1% of the annual risk of infection in the overall population (de March-Ayuela P, 1994). Adults with infectious pulmonary tuberculosis are the greatest danger to children, as they are the source of most childhood TBM and successful adult treatment programmes have been shown to reduce the incidence of childhood TBM (Zhang LX *et al.*, 2000a).

In populations with lower tuberculosis prevalence, most cases of TBM are in adults. Recent series suggests that an increasing proportion of these adults are immigrants from areas of high prevalence for tuberculosis (Bidstrup C *et al.*, 2002). Risk factors

also identified for the development of TBM are alcoholism, diabetes mellitus, malignancy and recent corticosteroid use (Davis LE *et al.*, 1993; Mori MA *et al.*, 1992; Pablos-Mendez A *et al.*, 1997). However, co-infection with HIV now dwarfs these risk factors. HIV increases the lifetime risk of developing clinical tuberculosis post-infection to 1 in 3 (Selwyn PA *et al.*, 1989), and in particular, predisposes to the development of extra-pulmonary tuberculosis and TBM (Bishburg E *et al.*, 1986). The risk increases as the CD4 count declines (De Cock KM *et al.*, 1992) and the disease constitutes either reactivation of latent infection, or new infection.

Evidence of genetic variability in human susceptibility to tuberculosis has been difficult to obtain. There are data to suggest some races are more susceptible to tuberculosis infection than others (Stead WW *et al.*, 1990) and more recent reports have focused upon specific allelic polymorphisms associated with the disease. Case-control association studies have recently revealed several polymorphisms in the NRAMP1 gene and the vitamin D receptor gene that are associated with human susceptibility to tuberculosis (Bellamy R *et al.*, 1998; Wilkinson RJ *et al.*, 2000). Immunological and genetic studies support the notion that innate immunity may be important in the control of *M.tb* (van Crevel R *et al.*, 2002), and an inadequate early innate immune response represents one hypothesis to explain the haematogenous dispersal of *M.tb* from the lung to other tissues like the brain. However, the extent to which an individual's genetic constitution affects resistance or susceptibility to infection remains uncertain (Bloom BR *et al.*, 1998) and there are no data to suggest genetic susceptibility to cerebral tuberculosis.

Whether BCG vaccination affords protection against pulmonary tuberculosis is still debated. A meta-analysis of the published trials on the efficacy of BCG vaccination suggested a protective effect against TBM of 64% (Colditz GA *et al.*, 1994). This figure is higher than that suggested for pulmonary tuberculosis (50%), but may only reflect more accurate case ascertainment of TBM given the universal requirement for hospitalisation. There are data from populations in which BCG vaccination has been withdrawn that suggests stopping vaccination has had no impact of the incidence of TBM (Zhang LX *et al.*, 2000b). However, it is likely that BCG vaccination protects against haematogenous dissemination of *M.tb* in childhood, in particular against miliary and cerebral tuberculosis.

1.2.2 Causative agent of tuberculous meningitis

The characteristics of *M.tb* enabling it to cause disease are complex and incompletely understood. Evidence that clinical isolates of *M.tb* have different biological characteristics has long been available. As discussed earlier, Riley demonstrated that guinea pigs exposed to air vented from the hospital rooms of tuberculosis patients showed remarkable variations in transmission (Riley RL *et al.*, 1961). The variations may be explained by differences in environment, infectious burden and host immunity. Experimental evidence suggests that the virulence of individual strains is also significant and selected gene mutations have been shown to affect virulence (Collins DM *et al.*, 1995; Valway SE *et al.*, 1998).

More recently, particular strains of tuberculosis have been associated with high rates of person-to-person transmission (Valway SE *et al.*, 1998) and possibly with cerebral tuberculosis (Arvanitakis Z *et al.*, 1998). There are, however, few data on the molecular characteristics of *M.tb* strains associated with different clinical phenotypes or outcomes. The determination of the complete genomic sequence for *M.tb* should accelerate research and understanding (Cole ST *et al.*, 1998) and future opportunities for research in this area are discussed in **Chapter 8.**

1.2.3 Pathogenesis of tuberculous meningitis

The pathogenesis of TBM can be viewed on two levels: macroscopically there are the mechanisms by which the bacilli disseminate to the CNS; microscopically there are the cellular and immune mechanisms that can result in the disease and its control.

The macroscopic development of TBM is a two-step process (Rich AR et al., 1933). Bacilli enter the host lung by droplet inhalation, and invade the pulmonary alveolar macrophage. Local infection escalates within the lung and disseminates to the regional lymph nodes producing the 'primary complex' characterised by the chest X-ray appearances of hilar lymph node enlargement and evidence of peripheral pulmonary infection (**Figure 1.3**). During this stage there may be a short but significant bacteraemia that can seed tubercle bacilli to other organs in the body. In those who develop TBM, bacilli seed to the meninges or brain parenchyma, forming small subpial or subependymal foci. These are called Rich foci, after the author of the original pathological studies describing this sequence of events (Rich AR et al., 1933). In approximately 10% of cases, particularly in children, the primary complex

does not heal but progresses to 'primary progressive tuberculosis'. Tuberculous pneumonia develops with heavier and more prolonged bacillaemia, and dissemination to the brain is more likely.

The second step in the development of TBM is rupture of a Rich focus into the subarachnoid space. This heralds the onset of meningitis, which if left untreated, will result in severe and irreversible neurological pathology. In 75% of children the onset of TBM is less than 12 months after the primary infection (Lincoln EM *et al.*, 1960). This period is believed to be much longer in adults. Three processes produce the subsequent neurological pathology: adhesion formation, an obliterative vasculitis and an encephalitis or myelitis (Dastur DK *et al.*, 1995). Adhesions result from a dense basal meningeal exudate that develops following inoculation of bacilli into the subarachnoid space. The exudate contains lymphocytes, plasma cells and macrophages, with increasing quantities of fibrin. Blockage, through adhesion formation, of the basal subarachnoid cisterns can result in obstruction of the cerebrospinal fluid (CSF) and hydrocephalus (**Figure 1.10**). Adhesions around the interpendicular fossa and related structures can compromise cranial nerves, particularly III, IV and VI, and the internal carotid artery. An obliterative vasculitis of both large and small vessels develops that can result in infarction and stroke syndromes (**Figure 1.11**). These commonly occur in the territories of the internal carotid artery, proximal middle cerebral artery and the perforating vessels to the basal ganglia (Hsieh F Y *et al.*, 1992). Infarction through vasculitis is the mechanism by which many of the diverse clinical neurological abnormalities in TBM occur, and accounts for an appreciable part of the irreversible neurological sequelae.

Figure 1.10 Gross hydrocephalus secondary to TBM (MRI) (HTD)

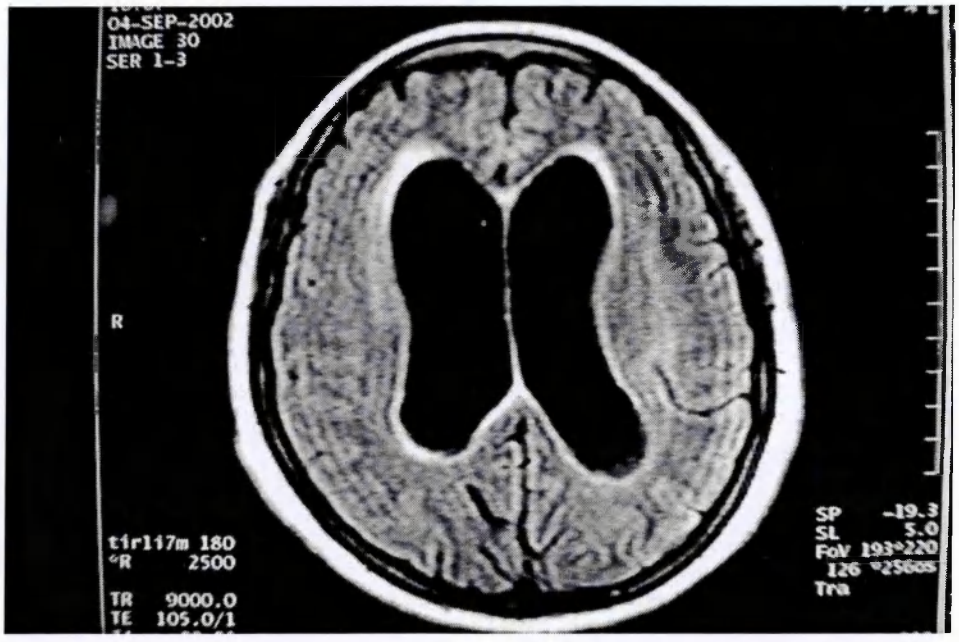
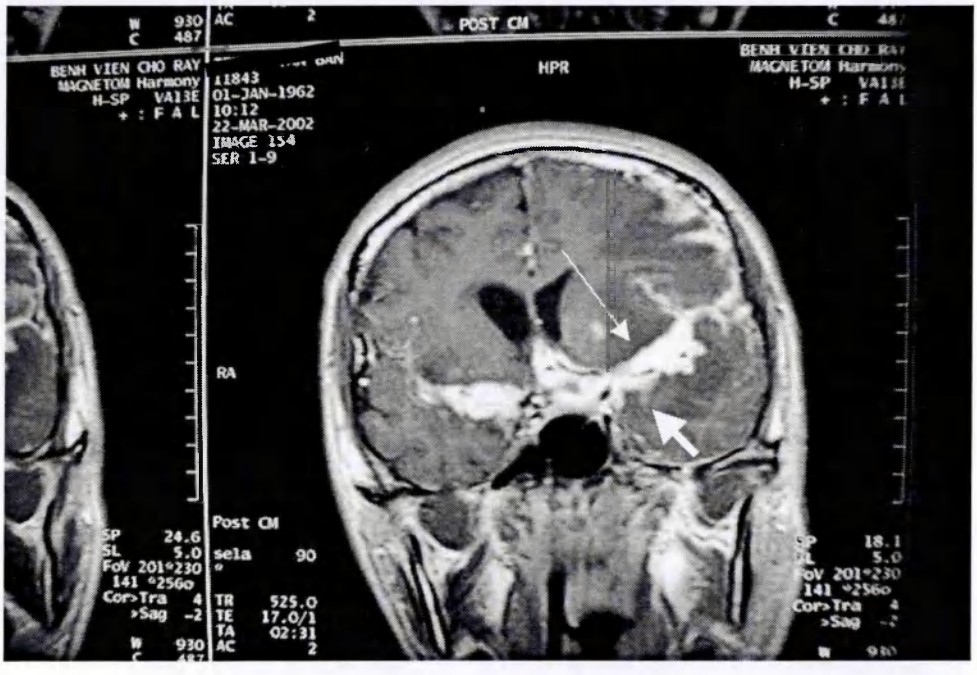


Figure 1.11 Infarction (thin arrow) **secondary to extensive inflammatory exudates** (thick arrow) **compromising the left middle cerebral artery** (MRI) (HTD)



The intensity of the basal inflammatory process extends into the parenchyma resulting in encephalitis. Oedema, occurring as a consequence, can be marked throughout both hemispheres, and will contribute to rising intra-cranial pressure and the global clinical neurological deficit.

The pathogenesis of TBM at the cellular and molecular level is poorly understood. Knowledge regarding the pathogenesis of pulmonary infection is limited, but may serve to illustrate some of the processes evident in the central nervous system (CNS). Theories of tuberculosis immuno-pathogenesis try to explain the roles and interactions between macrophage, T-helper cell and *M.tb*. Cell-mediated immunity is central to both the control of infection and the production of tissue damage (Dannenberg AM, Jr., 1991). Lurie's experiments on tuberculosis in rabbits described the fundamental stages of the disease (Lurie MB, 1964), and theories of immuno-pathogenesis aim to explain these stages.

The initial stage of infection is the ingestion of the inhaled tubercle bacilli by the alveolar macrophage. The bacilli multiply, and destroy the macrophage, depending on the ability of the macrophage to resist infection. The innate and possibly genetically determined resistance to infection at this stage has been discussed earlier. During the second stage bacilli grow logarithmically within newly recruited macrophages (Dannenberg AM, Jr., 1991). After approximately 2 weeks CD4 T-cells specific for mycobacterial peptides appear. Activated macrophages produce interleukin 1-beta, and tumour necrosis factor- alpha (TNF-α) that promote granuloma formation and enhance bacterial killing (Flynn JL *et al.*, 2001).

The primary complex results in the development of cell-mediated immunity; therefore

the rupture of the Rich focus, with release of bacilli into the subarachnoid space, probably results in a local T-cell dependant response. This response is characterised macroscopically as caseating granulomatous inflammation (Dastur DK *et al.*, 1995). Dannenberg hypothesizes that necrosis is the result of a delayed-type hypersensitivity reaction to exposed tuberculoproteins (Dannenberg AM, Jr., 1991). Others propose that the granuloma architecture dictates the extent of the central necrosis: a reduced capacity for focusing T-lymphocytes within a point of infection may result in failure to deliver adequate cytokine concentrations to the centres of large granulomas, resulting in degeneration and necrosis (Orme IM, 1998; Turner OC *et al.*, 2003).

The intra-cerebral immune response to *M.tb* is at least in part dependent on the production and regulation of cytokines. *M.tb* induces TNF-α secretion from macrophages, dendritic cells and T-cells and is required for successful control of infection (Flynn JL *et al.*, 2001). In particular, TNF-α is considered to be critical for granuloma formation (Flynn JL *et al.*, 1995), but is also cited as a major factor in host-mediated destruction of infected tissue (Rook GA *et al.*, 1987). Small concentrations of TNF-α given to animals previously exposed to *M.*tb result in substantial tissue necrosis (Filley EA *et al.*, 1991). Studies in bacterial meningitis show CSF levels of TNF-α correlated with disease severity (Sharief MK *et al.*, 1992). Rabbit models of TBM show CSF concentrations of TNF-α correlated with clinical progression (Tsenova L *et al.*, 1999), and intervention with antibiotics and thalidomide, an anti-TNF-α agent, resulted in an improvement in survival and neurological outcome (Tsenova L *et al.*, 1998).

However, CSF TNF-α concentrations in TBM from human subjects are lower than in human bacterial meningitis (Akalin H *et al.*, 1994) or in the rabbit TBM model and have not been correlated with disease severity or outcome. Studies in humans have been small and have lacked the statistical power to confirm associations between any CSF cytokine and disease severity and outcome. Clearly, the roles of TNF-α in tuberculosis protection and disease are complex and incompletely understood.

Although a protective response to *M.tb* is dependent on cell-mediated immunity, little is known about the cells present in the CSF in those with TBM and their role in pathogenesis. Clinical series attest to the difference in CSF cellular responses between bacterial and tuberculous meningitides: CSF cell counts in bacterial meningitis are frequently greater than 5000 x 10^3 white cells/ml, but in TBM they are rarely greater than 1000 x 10^3 white cells/ml. Few studies have attempted to phenotype the cells beyond the basic divisions of neutrophil and lymphocyte. The limited available data suggests many of the lymphocytes are CD4+ T-helper cells (El-Naggar A *et al.*, 1981), although more recent evidence suggests gamma delta (γδ) T-cells (CD4 and CD8 negative) may be important (Dieli F *et al.*, 1999). Further characterisation of the cells, cytokines, and chemokines found in the CSF from patients with TBM and how they change with treatment is required to increase understanding of disease pathogenesis.

It is also clear that other classes of compounds may be important to pathogenesis, in particular the matrix metalloproteinases. These molecules, secreted by monocytes and macrophages, are zinc-containing proteases that degrade extra-cellular matrix (Goetzl EJ *et al.*, 1996). They may cause cerebral injury by disrupting the blood brain barrier

(BBB), facilitating leukocyte migration, and by cleaving myelin proteins. Elevated CSF matrix metalloproteinase-9 (MMP-9) concentrations have been associated with focal neurological deficit and fatal outcome in Vietnamese adults with TBM (Price NM *et al.*, 2001).

1.2.4 Clinical features of tuberculous meningitis

Physicians find the diagnosis of TBM difficult because the clinical features are variable and non-specific. These features have been extensively described in a multitude of case reports and clinical series and are similar to many sub-acute meningo-encephalitides (Davis LE *et al.*, 1993; Farinha NJ *et al.*, 2000; Girgis NI *et al.*, 1998; Kent SJ *et al.*, 1993; Verdon R *et al.*, 1996). Diagnostic uncertainty commonly arises in a comatose patient presenting with a few days of headache, fever and neck-stiffness; undefined treatment in the community, and a CSF containing mostly lymphocytes, with a low glucose.

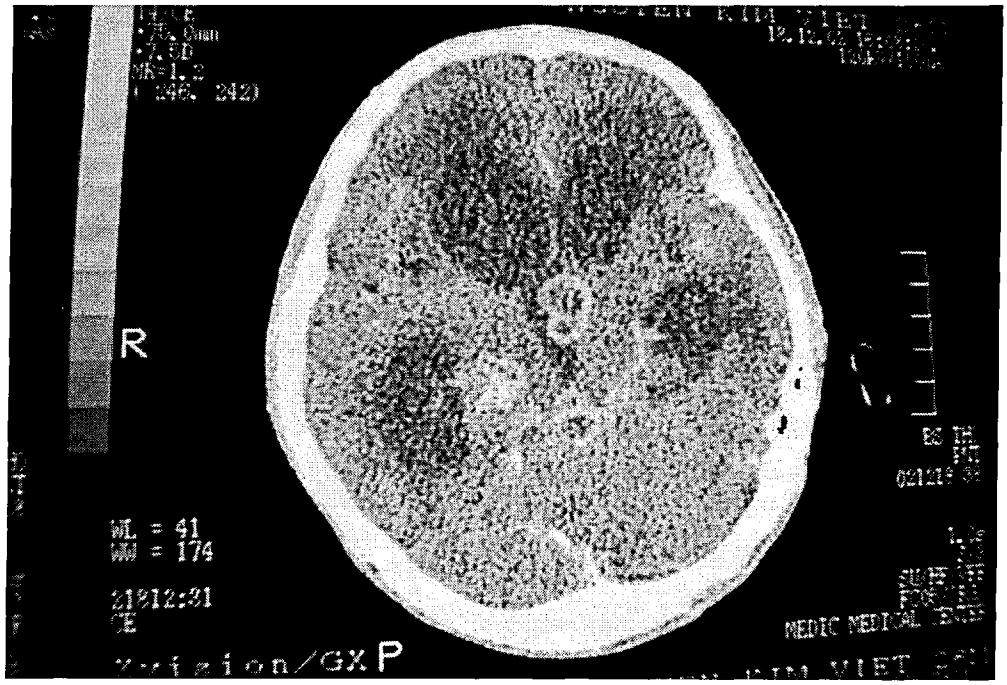
The patient's description of the onset and variety of symptoms is often unhelpful. One series reported that on admission to hospital only 28% complained of headache, 25% were vomiting, 13% reported fever, and 2% described the classical meningitic symptoms of photophobia and neck stiffness (Kent SJ *et al.*, 1993). As a consequence, TBM was considered a diagnosis in 36% of cases, with only 6% receiving immediate treatment. A history of recent contact with tuberculosis may be more helpful, particularly in children: several studies report 50-90% of children recalled recent contact with an adult with tuberculosis (Donald PR *et al.*, 1998) (Farinha NJ *et al.*, 2000).

The neurological complications of TBM are legion (Leonard JM *et al.*, 1990). Their nature and diversity can be predicted from the site of disease and the pathogenesis. Adhesions can result in cranial nerve palsies (particularly II, III, IV, VI, VII), or constriction of the vessels resulting in stroke. Obstruction of CSF flow leads to raised intra-cranial pressure, hydrocephalus and reduced conscious level (**Figure 1.12 and 1.13**). Infarcts occur in approximately 30% of cases (Tartaglione T *et al.*, 1998), commonly in the internal capsule and basal ganglia, causing a range of problems from hemiparesis to movement disorders.

Seizures are more common in children than adults, and may be caused by hydrocephalus, tuberculoma, oedema and hyponatraemia due to inappropriate anti-diuretic hormone secretion. The diagnosis of spinal meningitis should be considered in those presenting with root pain, with either spastic or flaccid paralysis and loss of sphincter control.

Figure 1.12 Right Lateral rectus palsy (VIth cranial nerve) in young woman with TBM (HTD)

Figure 1.11 Hydrocephalus and multiple tuberculomas in adult with TBM (CT with contrast) (HTD)



Diagnosis is dependent on lumbar puncture and CSF examination. Abnormalities in the CSF depend upon a tuberculin reaction within the subarachnoid space. The usual findings are of between 100 – 1000 cells/mm3 in the CSF: the majority are normally lymphocytes, although neutrophils may predominate early in the disease (Jeren T *et al.*, 1982). Those with depressed cell-mediated immunity may have atypical findings, and acellular CSF is reported in elderly and HIV positive patients (Karstaedt AS *et al.*, 1998; Laguna F *et al.*, 1992). An elevated CSF protein occurs in the majority and CSF glucose will be reduced in 70% or more (Verdon R *et al.*, 1996).

Over the last 10 years there have been a number of studies documenting the relationship between HIV and TBM (Berenguer J *et al.*, 1992; Dube MP *et al.*, 1992;

Yechoor VK *et al.*, 1996). These reports suggest HIV infected patients are at increased risk of TBM, but the clinical features and outcomes of the disease are similar. However, concomitant extra-meningeal disease may be more common: in one report 77% with HIV had clinical evidence of extra-meningeal tuberculosis, compared with 9% without HIV (Karstaedt AS *et al.*, 1998). Cerebral tuberculoma may also be more common in those infected with HIV (Dube MP *et al.*, 1992). These characteristics may help suggest the diagnosis of TBM in those with HIV.

1.2.5 Prognosis of tuberculous meningitis

A number of studies have assessed the clinical and laboratory parameters that might predict outcome. The first studies used univariate analysis to suggest extremes of age, advanced stage of disease, concomitant extra-meningeal tuberculosis, and evidence of raised intra-cranial pressure were associated with a poor outcome (Gulati PD *et al.*, 1970). Later studies have adjusted for the effect of co-variables using multivariate analyses and have consistently shown that treatment before the onset of coma improves outcome. A retrospective study of 434 Turkish adults revealed convulsions, coma and delayed or interrupted treatment to be independent predictors of mortality (Hosoglu S *et al.*, 2002). Extra-meningeal tuberculosis, cranial nerve palsy, focal weakness, multiple neurological abnormalities and drowsiness independently predicted later neurological disability.

The message for physicians is simple – they must not delay treatment in those in whom the diagnosis is suspected, although the limitations of current diagnostic methods will result in many patients starting treatment without a confirmed diagnosis.

1.2.6 Diagnosis of tuberculous meningitis

The diagnosis of TBM is difficult regardless of the resources available to the physician. As untreated TBM is almost always fatal it is essential that any diagnostic test is sensitive. The test must also be rapid, because a poor outcome is strongly associated with delayed treatment (Hosoglu S *et al.*, 2002). At present, no diagnostic method satisfies both these requirements. The methods available are limited, despite the wealth of possibilities suggested in the literature.

Clinical diagnostic methods

Few studies have attempted to define exactly which clinical features are predictive of the diagnosis of TBM. In one, five presenting clinical features were found to be independently predictive of the diagnosis in 232 children: prodromal stage ≥ 7 days, optic atrophy, focal neurological deficit, abnormal movements and CSF leucocytes < 50% polymorphs (Kumar R *et al.*, 1999). The authors developed a simple diagnostic rule: when at least one feature was present diagnostic sensitivity was 98%, specificity 44%; if three or more features were present sensitivity was reduced to 55% but specificity rose to 98%. However, there may be problems applying this rule: if one feature is taken to be diagnostic more than half will be given ATC unnecessarily, but if three features are required nearly half with TBM will not be treated. The consequences of the former will be unnecessary toxicity, and death in the later. Furthermore, the performance of this rule, and others like it, will depend upon the prevalence of tuberculosis in the population in which they are used.

Direct CSF examination and culture for acid-alcohol fast bacilli

The search for AFB in clinical specimens has remained the cornerstone of diagnosis ever since Robert Koch first saw the bacillus in 1882. In 1953 Stewart described the method by which her laboratory demonstrated acid-alcohol fast bacilli in 91 of 100 consecutive cases of TBM, all of which were subsequently confirmed by culture (Stewart SM, 1953). Similar results were reported more recently by Kennedy, who found that bacilli were present in the CSF of 45/52 (87%) patients with a clinical diagnosis of TBM (Kennedy DH *et al.*, 1979). However, many laboratories find these results difficult to reproduce. The sensitivity of the direct smear may depend upon the volume of CSF: 10-20ml were examined by Stewart, and as many as four specimens by Kennedy. Clinicians may be reluctant to take the volumes of CSF required to demonstrate the bacilli. Also, the laboratory must undertake a meticulous search for AFB. Stewart suggested a slide should be examined for between 30 and 120 minutes, a feat that few modern laboratories would have the staff or patience to complete. An understanding of the factors that govern the performance of conventional bacteriology is required.

Although culture of CSF for *M.tb* is the diagnostic 'gold standard' for TBM, it takes too long for early diagnosis and treatment. The factors that govern sensitivity are probably the same as for direct smear and diagnostic confirmation by culture can be extremely helpful in patients who have been started on ATC on clinical grounds alone. In most circumstances the sensitivity and specificity of culture will exceed that

of direct smear. However, once treatment has been started the sensitivity of culture falls quickly. AFB (presumed to be dead) can be found in the CSF for some days after the start of treatment even though they will not grow in culture (Kennedy DH *et al.*, 1979).

CSF adenosine deaminase activity

The activity of adenosine deaminase, an enzyme produced by CD4+ lymphocytes and monocytes, is raised in the CSF of patients with TBM. A number of studies have evaluated this as a diagnostic assay (**Table 1.2**). The enzyme activity cut-off value used by each study varied from 4 – 10 IU/ml. Diagnostic sensitivity ranges between 44 and 100%, and specificity between 71 and 99%. There are three important problems with this assay.

First, there is no accepted diagnostic cut-off for the levels of CSF enzyme activity. Second, the assay has not been evaluated in those with HIV infection, which depletes adenosine deaminase producing T-lymphocytes and may reduce diagnostic sensitivity. Third, patients with lymphomas, malaria, brucellosis and pyogenic meningitides also have high levels of CSF adenosine deaminase activity. In particular, the clinical and laboratory features of partially treated pyogenic meningitis commonly cause diagnostic confusion with tuberculous meningitis - an assay that cannot reliably discriminate between these two conditions is not useful.

Table 1.2 Studies of CSF adenosine deaminase activity for the diagnosis of TBM

Study	Case mix	Cut-off (IU/ml)	Sensitivity	Specificity
(Mann MD *et al.*, 1982)	Suspected TBM only	TBM >5	85%	84%
(Coovadia YM *et al.*, 1986)	Unselected	TBM >10	73%	71%
(Ribera E *et al.*, 1987)	Unselected	TBM ≥9	100%	99%
(Rohani MY *et al.*, 1995)	Unselected	TBM > 9	100%	88%
(Mishra OP *et al.*, 1995)	TBM and partially treated bacterial meningitis	TBM ≥ 5	63%	89%
(Lopez-Cortes LF *et al.*, 1995)	Unselected	TBM ≥10	50%	96%
(Mishra OP *et al.*, 1996)	Unselected	TBM ≥ 5	89%	92%
(Baro M *et al.*, 1996)	Unselected	TBM > 6.5	83%	85%
(Gambhir IS *et al.*, 1999)	Unselected	TBM ≥ 8	44%	75%

The detection of Mycobacterium tuberculosis nucleic acid in the CSF

The amplification and detection of *M.tb* nucleic acid from CSF is an attractive diagnostic prospect. Diagnostic specificity is dependent on selecting a region of the genome unique to *M.tb* while sensitivity is enhanced by amplification of the selected region from a clinical specimen. This method would appear to be particularly suitable

Table 1.3 Amplification and detection of nucleic acid for the diagnosis of tuberculous meningitis

STUDY	TARGET	GOLD STANDARD	SENSITIVITY	SPECIFICITY
(Kaneko K *et al.*, 1990)	MBP64	Clinical	(5/6) 83%	(20/20) 100%
(Shankar P *et al.*, 1991)	MPB 64	Clinical	(22/34) 65%	(45/51) 88%
(Machado LR *et al.*, 1994)	65KDa antigen	Clinical	(7/10) 70%	(10/10) 100%
(Lee BW *et al.*, 1994)	MPB 64	Clinical	(5/6) 83%	(12/13) 92%
(Folgueira L *et al.*, 1994)	IS6110	Clinical	(8/8) 100%	(14/14) 100%
(Liu PY *et al.*, 1994)	MPB 64	Clinical	(19/21) 90%	(79/79) 100%
(Lee BW *et al.*, 1994)	1)IS6110 2)MPB 64 3)65 KDa	Clinical	1)(6/6) 100% 2)(5/6) 83% 3)(5/6) 83%	(8/21) 38% (19/21) 90% (14/21) 67%
(Kox LF *et al.*, 1995)	IS6110	Clinical	(11/23) 48%	(19/19) 100%
(Miorner H *et al.*, 1995)	IS6110	Clinical	(18/33) 54%	(32/34) 94%
(Scarpellini P *et al.*, 1995)	IS6110	Culture and autopsy	(17/17) 100%	(24/24) 100%
(Lin JJ *et al.*, 1995)	MBP64	Clinical	(14/20) 70%	(26/27) 96%
(Seth P *et al.*, 1996)	MBP 64	Clinical	(34/40) 85%	(46/49) 94%
(Nguyen LN *et al.*, 1996)	IS6110	Clinical	(27/97) 28%	(36/39) 92%
(Pfyffer GE *et al.*, 1996)	rRNA[c]	Clinical	(54/54) 100%	(52/54) 97%
(Bonington A *et al.*, 1998)	16SrRNA[a]	Clinical	(10/40) 25%	(29/29) 100%
(Lang AM *et al.*, 1998)	rRNA[c]	Clinical	(8/24) 33%	(60/60) 100%
(Wei CY *et al.*, 1999)	IS6110 + MPB 64	Clinical	(3/5) 60%	(4/6) 67%
(Caws M *et al.*, 2000)	IS6110	Clinical	(9/23) 39%	(107/108) 99%
(Bonington A *et al.*, 2000)	16SrRNA[b]	Clinical	(7/35) 20%	(31/31) 100%
(Narayanan S *et al.*, 2001)	1)IS6110 2)TRC_4	Clinical	1)(54/67) 81% 2)(61/67) 91%	(23/29) 79% (22/29) 76%

[a] commercial Roche AMPLICOR kit
[b] commercial Roche COBAS AMPLICOR kit
[c] commercial 'Amplified *M.tuberculosis* Direct test' (MTD)

for the diagnosis of TBM when there are few tubercle bacilli in the CSF and a low chance of contamination with other bacteria. However, despite the development of commercially available assays, the promise is not yet matched by consistent diagnostic performance. **Table 1.3** shows the DNA or RNA target sequence, diagnostic gold standard, and sensitivity and specificity of 20 studies evaluating the use of the nucleic acid amplification (NAA) tests for the diagnosis of TBM. The variety of targets and 'gold standard' diagnostic criteria is a major impediment to comparing these studies. Few studies have carefully compared the sensitivity of NAA, smear and culture using large volumes of CSF. Those that have suggest the sensitivity of CSF smear is similar to NAA (Bonington A *et al.*, 2000). However, once treatment has begun NAA may be more helpful as the sensitivity of both smear and culture fall sharply - mycobacterial DNA remains detectable within the CSF for up to one month after the start of treatment (Donald PR *et al.*, 1993).

The variable specificity of NAA tests may arise for a number of reasons. False-positives may occur through cross-reaction with other mycobacteria, from contamination with other clinical samples, or from contaminating DNA in the laboratory (Noordhoek GT *et al.*, 1996). For this reason, a positive result should be placed within the clinical context before starting treatment, and laboratories must be committed to rigorous internal and external quality control of the use of the assay in different clinical specimens. This is time-consuming and expensive and is probably only possible within defined diagnostic reference centres.

The tuberculin skin test

The value of the tuberculin skin test for the diagnosis of TBM varies according to age, vaccination with BCG, nutritional status, HIV infection and the prevalence of tuberculosis. Diagnostic sensitivity is most commonly compromised by the phenomenon of 'anergy' - the failure of those with known infection with *M.tb* to respond to intra-dermal injection of tuberculin. These false negative results occur most commonly in elderly, malnourished patients with disseminated tuberculosis and in those with HIV infection i.e. those most at risk of developing TBM. Reports suggest only 20% have a positive tuberculin test (Girgis NI *et al.*, 1998). However, skin testing may be more useful in children: a South African series reported 86% had greater than 15mm of induration with 5 Units of tuberculin (Donald PR *et al.*, 1998). Positive results must also be seen within the context of the tuberculosis prevalence in the area. Individuals from high prevalence areas are more likely to have positive tests with an unrelated illness. The limitations must be appreciated before interpreting the results of a skin test. The test is never diagnostic of TBM, but it can be helpful when assessing the likelihood of disease.

The chest X-ray and brain imaging

About one half of patients with TBM have a chest X-ray suggesting active or previous pulmonary tuberculosis (Girgis NI *et al.*, 1998). But, in areas of high tuberculosis prevalence radiological evidence of previous pulmonary infection is common, and the finding must be interpreted with caution. A proportion of patients with TBM (~10%) have a miliary chest X-ray appearance (**Figure 1.14**).

A number of studies have investigated the role of brain computerised tomography (CT) - hydrocephalus and contrast enhancing exudates in the basal cisterns are the most common findings (**Figure 1.15**) (Bhargava S *et al.*, 1982; Bullock MR *et al.*, 1982; Hsieh FY *et al.*, 1992; Kumar R *et al.*, 1996; Ozates M *et al.*, 2000; Teoh R *et al.*, 1989). However, as autopsy studies have also shown, hydrocephalus is more common in children than adults - brain CT demonstrates severe hydrocephalus in 87% of children, but only 12% of adults (Bhargava S *et al.*, 1982). Kumar compared the CT appearances of 94 children with TBM and 52 with pyogenic meningitis.

Figure 1.14 Chest X-ray showing miliary tuberculosis (HTD)

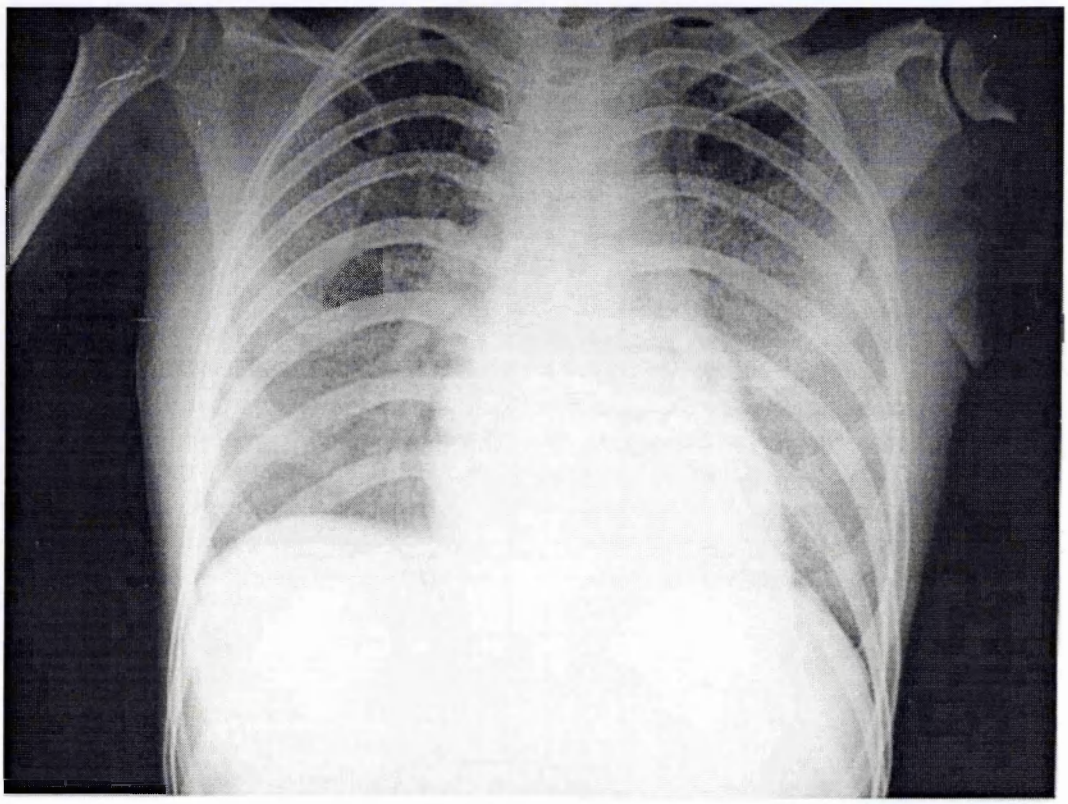
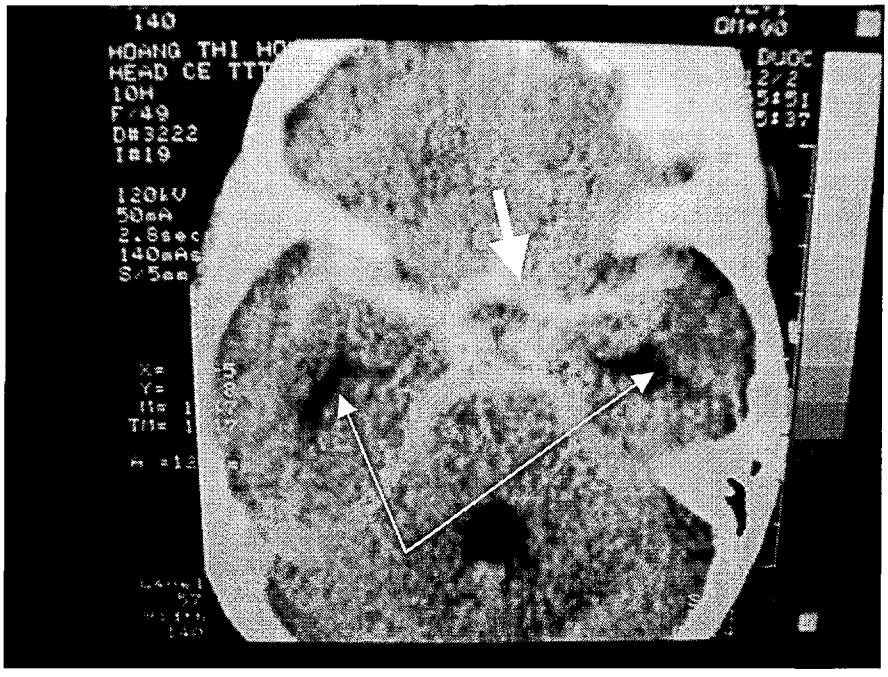


Figure 1.15 CT brain (with contrast) showing basal meningeal enhancement (thick arrow) **and hydrocephalus** ((thin arrows) (HTD)



Basal meningeal enhancement, tuberculoma, or both, were 89% sensitive and 100% specific for the diagnosis of TBM (Kumar R *et al.*, 1996). Teoh suggested, "a normal scan in a drowsy patient excludes the diagnosis of tuberculous meningitis" (Teoh R *et al.*, 1989). However, in a recent series the scan was normal in 35 of 289 (12%) patients with TBM and not all were fully conscious (Ozates M *et al.*, 2000). The abnormalities in this series were hydrocephalus (80% of children, 43% of adults), parenchymal enhancement (26% of children, 8% of adults), contrast enhancement of basal cisterns (15% of children, 23% of adults), cerebral infarct and focal or diffuse brain oedema (14% of children, 13% of adults) and tuberculoma (4% of children, 7% of adults) (Ozates M *et al.*, 2000).

Cranial magnetic resonance imaging (MRI) may provide more diagnostic information than CT when assessing space-occupying lesions, infarcts and the extent of the inflammatory exudates (Offenbacher H *et al.*, 1991; Schoeman J *et al.*, 1988; Tartaglione T *et al.*, 1998). However, data regarding the diagnostic sensitivity and specificity of MRI for TBM are limited – those studies that do exist are small and only include a highly selected patient population. However, it is reasonable to conclude that MRI is more sensitive than CT for detecting the diverse cranial pathology of TBM, although neither will reveal appearances specific for the diagnosis of TBM. Cryptococcal meningitis, cytomegalovirus encephalitis, sarcoidosis, meningeal metastases, and lymphoma may all produce similar radiographic findings (**Figures 1.16 and 1.17**).

Figure 1.16 MRI of adult with TBM showing dilated ventricles and peri-ventricular enhancement (arrows) (HTD)

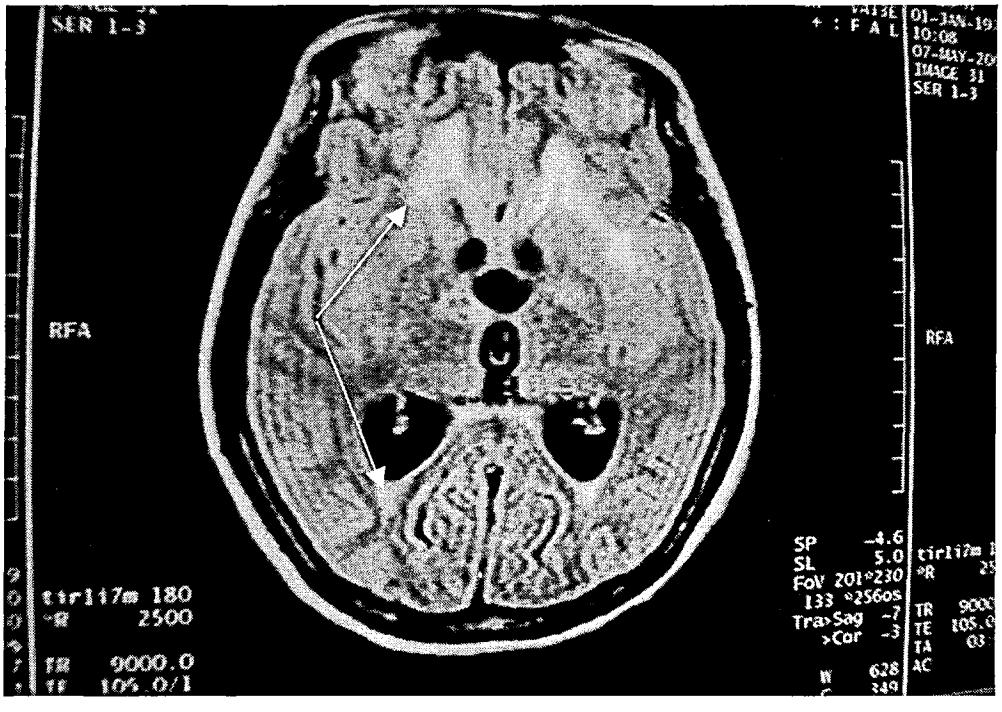
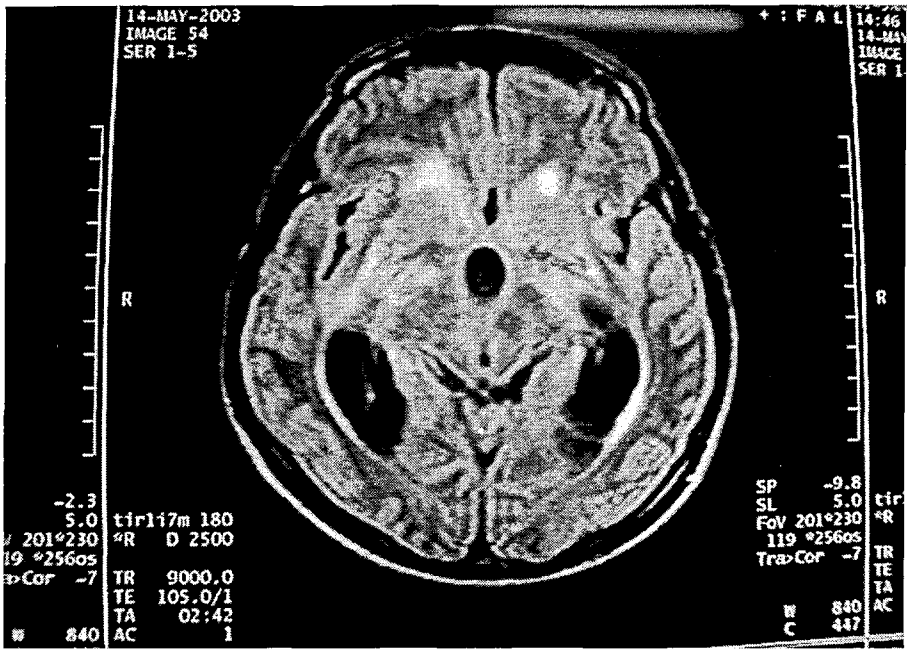


Figure 1.17 MRI of HIV negative adult with cryptococcal meningitis showing dilated ventricles and peri-ventricular enhancement mimicking TBM



Alternative diagnostic approaches

The challenge facing new diagnostic strategies in TBM is that they must improve on the sensitivity of conventional ZN staining and culture, but maintain the specificity. In the developed world cost is less critical, but in the developing world cost considerations mandate tests that are cheap, use standard reagents with long shelf lives that do not require refrigeration and are simple to perform.

Tuberculosteric acid (TSA) is a structural component of mycobacteria that was first detected in the CSF of a patient with TBM in 1983 (Mardh PA et al., 1983). Frequency pulsed electron-capture gas-liquid chromatography has been used to detect femtomole quantities of TSA in CSF, with reported sensitivity 91% and specificity 95% (Brooks JB et al., 1990). However, the cost of the equipment, and the

complexity of the technique mean it is unlikely to be adopted as standard diagnostic procedure.

Serological techniques that detect the intra-thecal synthesis of anti-mycobacterial antibodies have been studied over many years. Many have shown promise, but none have demonstrated consistent performance in a routine diagnostic laboratory. A good test will require an antigen with high species specificity and good immunogenicity. Enzyme-linked immunosorbent assays (ELISA) using crude antigens such as PPD have resulted in low sensitivity and specificity (Kalish SB et al., 1983; Watt G et al., 1988). The adaptation of ELISA techniques, and the identification of specific *M.tb* antigens have improved results. Using a solid phase antibody competition assay with mouse monoclonal antibodies (MABs) to the 38 kDA antigen (also known as antigen 5, or antigen 78), a large study was performed in pulmonary and extra-pulmonary TB (Wilkins EG et al., 1990). In extra-pulmonary tuberculosis diagnostic sensitivity was 73% and specificity 98%, regardless of organ site. More recently, the presence of IgG antibodies against six protein antigens (ESAT-6, 14kDa, 19kDa, MPT63, MPT64, 38kDa) were assessed by ELISA in the CSF of 442 patients with TBM and 102 controls (Chandramuki A et al., 2002). None of the controls had detectable IgG to any of the antigens. IgG to at least one antigen were detectable in 228/264 (87%) of HIV negative patients with clinical TBM, 50/69 (72%) with culture-proven TBM and 47/72 (65%) with autopsy-proven TBM. The CSF of more than 50% of TBM patients was reactive to all 6 antigens. There was some evidence of preferential antigen recognition according to clinical grouping: the culture confirmed cases reacted most strongly against the 14kDa, and antibodies against MPT64 were lowest in the CSF

from autopsy proven cases. Different stages of TBM may correlate with preferential recognition of different antigens, and disease progression may alter the antibody profiles. This study suggests future antibody detection diagnostic assays for TBM may have to assess reactivity to a range of antigens.

The differentiation of acute infection from previous exposure may be problematic in antibody detection tests, and test sensitivity may be compromised in immune compromised individuals. Methods to directly detect specific *M.tb* antigens in the CSF have been developed to tackle these inadequacies. Initial studies used a variety of ELISA techniques (Brooks JB *et al.*, 1990; Kadival GV *et al.*, 1986; Radhakrishnan VV *et al.*, 1990; Sada E *et al.*, 1983; Watt G *et al.*, 1988); the majority using polyclonal antibodies directed against crude antigen. Despite an expected lack of sensitivity and specificity, one retrospective study revealed a sensitivity of 68% and specificity of 100% using these components (Radhakrishnan et al.,1990). Other studies have claimed the identification of novel specific TB antigens and based specific serological tests on them. For example, 100% sensitivity (when compared with culture) and 100% specificity was reported by using a preparation of 35kDA *M.tb* antigen contained on nitrocellulose strips (Mathai A *et al.*, 1994). The test was simple to perform and the strips had a shelf life of 2 years. Unfortunately this and many similar assays have showed early promise in highly controlled studies, but have not performed so well in clinical practice.

1.2.7 Treatment of tuberculous meningitis

Anti-tuberculosis chemotherapy

The treatment of TBM follows the model of short course chemotherapy for pulmonary tuberculosis - an intensive phase of treatment, followed by a continuation phase. But unlike pulmonary tuberculosis, the optimal drug regimen and duration of each phase are uncertain.

Streptomycin was first used to treat tuberculosis in 1944, and in 1946 the UK MRC began studies using streptomycin for TBM. In 1948 they published data that demonstrated a marked improvement in outcome for those with TBM treated with streptomycin (MRC, 1948b). Mortality fell to 46% in those presenting with stage 1 (conscious, no neurological deficit), 66% in stage 2 (disturbed consciousness, with or without focal neurology) and 86% in stage 3 (comatose, with or without focal signs). The introduction of isoniazid and para-amino-salicylic acid (PAS) led to further improvements in prognosis. A report documenting the changes in available chemotherapy between 1947-1958 reveals mortality fell from 64% using streptomycin alone to 27% with streptomycin and PAS, then to 17% with the addition of isoniazid (Lorber J, 1960).

The addition of rifampicin to the treatment of TBM was immediately accepted although the prognostic benefits of rifampicin have been questioned (Ramachandran P *et al.*, 1986; Ramachandran P *et al.*, 1989), and uncertainty surrounds its penetration into the CSF. Rifampicin is 80% protein bound in plasma, enabling a maximum of 20% to penetrate the CSF in those with an intact BBB. Studies have shown slow penetration of rifampicin into the CSF of patients with TBM, with levels just above

the minimum inhibitory concentrations (MIC) for *M.tb* (Ellard GA *et al.*, 1993) (**Table 1.4**). Meningeal inflammation enhances CSF penetration of anti-tuberculosis drugs, although there is limited evidence to suggest rifampicin penetration occurs independently of inflammation (Nau R *et al.*, 1992).

There is no conclusive evidence to demonstrate improvement in outcome with the use of pyrazinamide. It is well absorbed orally, and has excellent penetration into the CSF (Forgan-Smith R *et al.*, 1973). These factors, and the sterilising effect on tubercle bacilli, have resulted in pyrazinamide being considered mandatory at the beginning of TBM treatment (BTS, 1998; Humphries M, 1992). It has been suggested that given the uncertain benefit and penetration of rifampicin, pyrazinamide should be given for the duration of the treatment (Donald PR *et al.*, 1998).

The British Thoracic Society (BTS) (BTS, 1998), the Infectious Diseases Society of America, and the American Thoracic Society (ATS) (ATS, 1994) recommend that all patients start on isoniazid, rifampicin, and pyrazinamide. Isoniazid is believed to be critical because it penetrates the CSF freely and has potent early bactericidal activity. Choosing the fourth drug of the intensive phase is more difficult. The BTS recommend either streptomycin or ethambutol, although neither penetrates the CSF well in the absence of inflammation, and both can produce significant adverse reactions. Streptomycin should not be given to those who are pregnant or have renal impairment. Intra-thecal streptomycin is no longer used, although this route of administration is being revisited for the treatment of multi-drug resistant cases.

Table 1.4 Studies of the CSF pharmacokinetics of the first-line antituberculosis drugs

Drug	Dose/24hrs	Route	No. patients	Inflamed meninges?	C max (mg/l)	T max (hours)	% of serum conc	Reference
Isoniazid	100 mg	Po	1	No	0.31	1	20	(Barclay WR *et al.*, 1953)
	1.5 mg/kg	Po	4	No	0.50-0.55	5.5	100	(Elmendorf DF *et al.*, 1952)
	1.8-3.1 mg/kg	Po	5	Yes	1.77-4.10	3.25-4.25	100	(Elmendorf DF *et al.*, 1952)
	600 mg	Po	1	Yes	2.0	3-6	>90	(Forgan-Smith R *et al.*,
	300 mg	Po	16	Yes	2.4	NK	90	1973)
	20 mg/kg	Po	38	Yes	12.0	2-4	95	(Kaojarern S *et al.*, 1991)
	9 mg/kg	Po	27	Yes	3.2	4	75	(Donald PR *et al.*, 1992)
								(Ellard GA *et al.*, 1993)
Rifampicin	600 mg	Po	13	No	0-0.81	3-9	0-5	(Kenny MT *et al.*, 1981)
	600 mg	Po	13	Yes	0.24-2.4	3-6	6-30	(Sippel JE *et al.*, 1974)
	600 mg	Po	10	Yes	1.06	6-12	20	(D'Oliveira JJ, 1972)
	600 mg	Po	12	Yes	0.3	8-10	10	(Woo J *et al.*, 1987)
	450-600 mg	Po	16	Yes	0.29	NK	5	(Kaojarern S *et al.*, 1991)
	600 mg	Iv	7	No	0.73	1	22	(Nau R *et al.*, 1992)
	11 mg/kg	Po	27	Yes	0.78	5	7	(Ellard GA *et al.*, 1993)
Pyrazinamide	3 g	Po	1	Yes	50	5	100	(Forgan-Smith R *et al.*,
	1.5-2.5 g	Po	12	Yes	42	5-6	100	1973)
	34-41 mg/kg	Po	28	Yes	44.5	5	100	(Woo J *et al.*, 1987)
	40 mg/kg	Po	13	Yes	50	2	No serum	(Ellard GA *et al.*, 1987)
	1.5 g	Po	17	Yes	50.2	NK	80-100	(Donald PR *et al.*, 1988)
	1.5 g	Po	16	Yes	35	NK	90	(Phuapradit P *et al.*, 1990)
								(Kaojarern S *et al.*, 1991)
Ethambutol	15-25 mg/kg	Po	10	No	0-1.9	NK	0-18.5	(Gundert-Remy U *et al.*,
	15-35 mg/kg	Po	17	Yes	0.3-4.21	NK	10-54	1973)
								(Bobrowitz ID, 1972)
Streptomycin	1g	Im	4	Yes	9	5	20	(Anderson DG *et al.*, 1945)
								(Forgan-Smith R *et al.*,
	0.75-1 g	Im	16	Yes	3.8	NK	20	1973)
	13 mg/kg	Im	27	Yes	2.2	2-6	7	(Kaojarern S *et al.*, 1991)
								(Ellard GA *et al.*, 1993)

Some centres, notably in South Africa, advocate ethionamide, which penetrates healthy and inflamed meninges (Donald PR *et al.*, 1989). However, despite theoretical attractions, there are no data demonstrating any clear advantage of one drug over another. The prevalence and patterns of drug resistance may influence the choice of drug. For example, streptomycin resistance is common (>25%) in Vietnam, particularly in the HIV population, and ethambutol is favoured as a consequence.

Isoniazid and rifampicin should be given throughout the continuation phase of treatment. Although there are no data to suggest pyrazinamide improves outcome, some authorities suggest it should accompany these drugs given the high CSF concentrations achieved throughout the course of the disease (Humphries M, 1992).

TBM caused by organisms resistant to both isoniazid and rifampicin (multi-drug resistance) presents a formidable therapeutic challenge. For suspected or proven multi-drug resistant pulmonary tuberculosis the World Health Organisation recommend an aminoglycoside (kanamycin, amikacin, or capreomycin), ethionamide, pyrazinamide, and ofloxacin for the initial phase of treatment (Crofton J *et al.*, 1997). There are no equivalent recommendations for multi-drug resistant TBM, and few data are available on the CSF penetration and effectiveness of possible agents. Ethionamide, prothionamide, and cycloserine have been reported to penetrate the blood-brain barrier and may be effective. The combination of intra-thecal amikacin and levofloxacin has also been suggested (Berning SE *et al.*, 2001). Until more data become available, the treatment of

patients with multi-drug resistant TBM should be guided by individual resistance profiles and the predicted CSF penetration of candidate drugs, although in many areas of the world suitable alternative drugs are not available.

The B TS recommendations for the dosages of the standard anti-tuberculosis drugs are shown in **Table 1.5**. Some authors have suggested using doses of isoniazid greater than 5 mg/kg for the treatment of TBM (Humphries M, 1992). The potent early bactericidal effect of isoniazid, and the uncertain CSF penetration of other drugs in the standard regimen, makes this an attractive suggestion. However, at standard doses isoniazid achieves CSF levels 10-15 times the minimum inhibitory concentration of *M.tb* (Kaojarern S *et al.*, 1991) (**Table 1.4**), and there are no data to suggest higher doses improve outcome or shorten treatment in adults with TBM.

TBM should be treated for at least six months. However, it is unclear whether more prolonged treatment is required. The BTS recommend 12 months in uncomplicated cases, extending to 18 months should pyrazinamide be omitted (BTS, 1998). Treatment for 12 months is probably an over-estimate of the time required for bacterial cure, and there is evidence to suggest shorter courses are effective. A recent systematic review concluded that six months of anti-tuberculosis drugs for TBM is probably sufficient, provided the likelihood of drug resistance is low (van Loenhout-Rooyackers *et al*, 2001). Disease severity, drug toxicity, and patient compliance should all be considered when deciding the duration of treatment.

Table 1.5 Recommended daily dosages of standard anti-tuberculosis drugs (BTS, 1998)

Drug	Children	Adults
Isoniazid	5 mg/kg	300 mg
Rifampicin	10 mg/kg	450 mg if weight < 50 kg
		600 mg if weight > 50 kg
Pyrazinamide	35 mg/kg	1.5 g if weight < 50 kg
		2.0 g if weight > 50 kg
Ethambutol	15 mg/kg	15 mg/kg
Streptomycin	15 mg/kg	15 mg/kg (max 1 g)

Adjunctive corticosteroids

The use of corticosteroids in the treatment of tuberculous meningitis is still controversial. The rationale lies in reducing the harmful effects of inflammation as the antibiotics kill the organisms, although corticosteroids do not appear to reduce the pro-inflammatory cytokines found in the CSF of those with TBM (Donald PR *et al.*, 1995). However, clinical trials suggest that corticosteroids may have a beneficial effect in some groups of patients and a consensus has emerged that adjuvant corticosteroids should be used in those presenting with MRC stage II or III TBM (BTS, 1998; Dooley DP *et al.*, 1997; Humphries M, 1992).

The limited evidence for this view is as follows. The first controlled trial to suggest benefit in using corticosteroids for TBM was published in 1955 (Ashby M *et al.*, 1955). Six out of 12 patients with TBM received steroids in addition to streptomycin and isoniazid. CSF white count fell faster in the steroid group, recovery from the acute phase was quicker, and none of the patients given steroids had any long-term sequelae. Four of the six who did not receive corticosteroids suffered chronic neurological sequelae. Trials with larger numbers were not performed until the mid 1970's when a prospective, randomised, double blind trial was performed in 72 patients (Escobar JA *et al.*, 1975). A reduction in mortality in the steroid group was shown, but the effect on neurological morbidity could not be assessed. The largest prospective, randomised, controlled trial to date enrolled 160 patients with TBM (Girgis NI *et al.*, 1991). Mortality and neurological sequelae were reduced in those treated with corticosteroids. The group that benefited the most were those with disease of intermediate severity. Those presenting either in a coma or with mild disease (MRC grades III and I) received minimal benefit.

Raised intra-cranial pressure (ICP) has long been considered important in the prognosis of TBM (Gulati PD *et al.*, 1970). Reduction of ICP by steroids was felt to be one of the means by which corticosteroids exerted their beneficial effect. A recent trial assessed the efficacy of steroids with regard to CT evidence of elevated ICP, parenchymal brain involvement, direct ICP measurements, and clinical outcome (Schoeman JF *et al.*, 1997). The trial showed no difference in ICP, ventricular size, or extent of infarction between those treated with or without steroids.

The benefit to mortality was again observed, as well as improved intellectual outcome in the steroid group. The data from six randomised controlled trials of corticosteroids for TBM, involving a total of 595 patients, has been subjected to a Cochrane review (Prasad K *et al.*, 2000). Those receiving corticosteroids had a lower death rate (relative risk 0.79, 95% Confidence Interval [CI] 0.65-0.97) and a reduced risk of death or severe neurological sequaelae (relative risk 0.58, 95% CI 0.38-0.88). Subgroup analysis suggested a beneficial effect on mortality in children, but inconclusive results in adults partly due to the small numbers of adult patients. The reviewers concluded that adjunctive corticosteroids may be of benefit in patients with TBM, but the evidence is limited. Existing studies are small with poor allocation concealment and publication bias may account for the results favouring corticosteroids.

There are no conclusive data regarding the choice of corticosteroid, dose or duration of treatment. A reduction in mortality has been suggested using either prednisolone or dexamethasone for at least the first three weeks of anti-tuberculosis chemotherapy, followed by a similar period, as the dose is decreased to zero (Girgis NI *et al.*, 1991; Schoeman JF *et al.*, 1997). These trials gave children 4mg/kg/day of prednisolone, or 12mg/day of dexamethasone, and adults' 16mg/day of dexamethasone.

It has been suggested that steroids may reduce the penetration of anti-tuberculous drugs into the CSF by reducing inflammation. There is little evidence for this occurring.

One study found no difference between the plasma/CSF level ratios of isoniazid, pyrazinamide, rifampicin, or streptomycin, in those on or off corticosteroids (Kaojarern S *et al.*, 1991).

Neurosurgery

Neurosurgical intervention may be indicated for hydrocephalus, tuberculoma, and abscess formation complicating TBM. Unfortunately, there are no published randomised trials of surgery for any of these complications. Hydrocephalus occurs more commonly in children than adults, but is the commonest reason for neurosurgical referral in both groups. A recent series reported that ventriculo-peritoneal shunting was performed in 30% (65/217) of children with hydrocephalus complicating TBM (Lamprecht D *et al.*, 2001). After six months of treatment 12% had died and 45% had severe sequelae. It is difficult to predict which patients with hydrocephalus will benefit from shunts. Clinical response to external ventricular drainage has been assessed for this purpose, but failed to predict benefit, so perhaps early ventriculo-peritoneal shunting should be considered in all patients with hydrocephalus (Mathew JM *et al.*, 1998; Palur R *et al.*, 1991). But this aggressive approach does not acknowledge the significant complications of shunt surgery, the variable resources and experience of surgical units, and the lack of trials demonstrating any benefit.

1.2.8 Response to treatment

Ninety percent of deaths from TBM occur in the first month of treatment (Girgis NI *et al.*, 1998). The response to treatment is usually slow and may fluctuate. Indeed, a rapid and sustained response over a few days suggests the diagnosis is wrong. The CSF mirrors the slow clinical response - cell counts are raised for 1-2 months, glucose remains low for a similar duration and total CSF protein can rise before falling slowly over many months (Schoeman JF *et al.*, 2001).

Transient episodes of high fever, worsening headache and increased neck rigidity can occur during the first two months of treatment, particularly in those with more severe disease. Distinguishing self-limiting events from the onset of more serious complications is difficult. Brain imaging should be arranged urgently if new clinical signs develop during treatment. Hydrocephalus, cerebral infarction, the expansion of intra-cranial tuberculoma (**Figure 1.18**), cerebral abscess formation (**Figure 1.19**) and poor adherence to therapy, are the foremost reasons for severe acute deterioration (Ranjan P *et al.*, 2003) (**Table 1.6**).

Table 1.6 Common reasons for clinical deterioration in patients with tuberculous meningitis.

Early deterioration (1-3 months)	**Late deterioration (3-12 months)**
1. Prescription error	1. Prescription error
2. Poor patient compliance	2. Poor patient compliance
3. Drug reaction requiring either reduction in dose, or withdrawal of all or some of the drugs	3. Paradoxical treatment reactions
4. Neurological drug reactions	
5. Paradoxical treatment reactions	

Paradoxical treatment reactions are a well-recognised phenomenon in all forms of tuberculosis. Although the pathogenesis remains unclear, such reactions are characterised by an intense inflammatory response. Data on the frequency and timing of these events in TBM are restricted to occasional case reports. The expansion of intra-cranial tuberculoma after the start of anti-tuberculosis drugs is the most widely reported example, and can occur at any time during treatment (Afghani B *et al.*, 1994). Most authors suggest treatment with prolonged high-dose corticosteroids, although there are no controlled trials to support this recommendation.

Figure 1.18 Intra-cranial tuberculomas developing after the start of ATC (MRI post contrast) (arrows) (HTD)

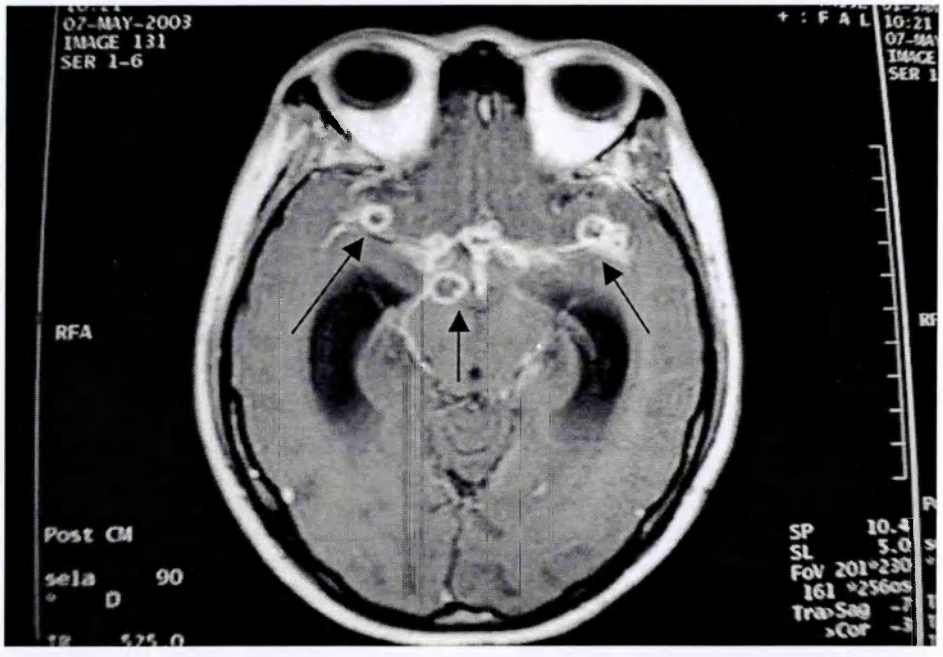
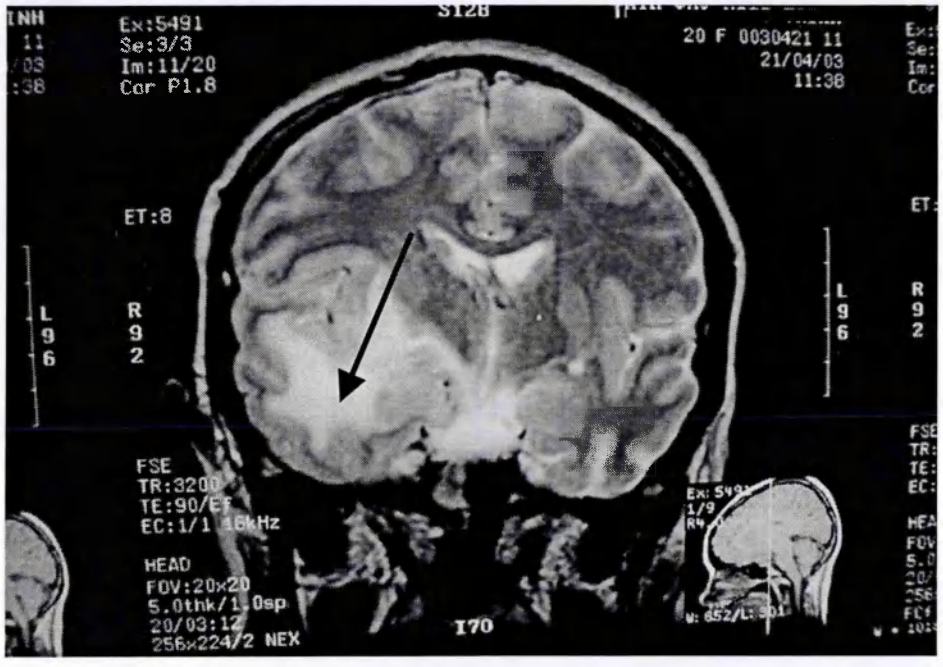


Figure 1.17 Tuberculous cerebral abscess (MRI) (arrow) (HTD)



Adverse reactions to ATC are a common problem, and can have a devastating effect on the outcome (Hosoglu S *et al.*, 2002). **Table 1.7** presents the common, the rare, and the neurological adverse reactions to the main antituberculosis drugs.

Table 1.7 Adverse reactions to antituberculosis drugs (BTS, 1998) (Holdiness MR, 1987)

Drug	Common	Rare	Neurological
Isoniazid	• Hepatitis	• haemolytic anaemia • aplastic anaemia • sideroblastic anaemia • agranulocytosis • lupoid reactions • arthralgia • gynaecomastia	• peripheral neuropathy • convulsions • optic neuritis • mania/psychosis
Rifampicin	• hepatitis • thrombocytopenia • fever	• haemolytic anaemia • acute renal failure	• headaches • confusion • drowsiness
Pyrazinamide	• hepatitis • anorexia • flushing • arthralgia • hyperuricaemia	• gout • photosensitivity	
Ethambutol	• arthralgia	• hepatitis • rash	• retrobulbar neuritis • peripheral neuropathy • confusion
Streptomycin	• vertigo • deafness • acute renal failure		• neuromuscular block

Hepatic toxicity is the commonest adverse event, and the BTS recommend stopping isoniazid, rifampicin, and pyrazinamide immediately if the transaminases rise to five times normal, or the bilirubin level rises (BTS, 1998). In most forms of tuberculosis a short period without treatment does not affect outcome. Unfortunately, treatment interruptions of this type in TBM are an independent predictor of death as they often lead to relapse with neurological deterioration (Hosoglu S *et al.*, 2002). Streptomycin and ethambutol should both be given in these circumstances, and isoniazid and rifampicin restarted as soon as possible. Treatment should be extended if the patient cannot adhere to the conventional nine-month regimen.

1.2.9 Summary

TBM is a difficult disease to diagnose and treat. The clinical features are non-specific, conventional bacteriology is widely regarded as too insensitive, and newer diagnostic methods are incompletely evaluated. Treatment relies on 4 drugs, all are more than 30 years old, and only prevents death in around 70%. *M.tb* resistant to these agents threatens to return TBM to the pre-chemotherapeutic era of 100% case fatality. Adjunctive treatment with corticosteroids might work, although it is not known how or why, and a sufficiently large controlled trial has yet to be completed. In short, clinical researchers have neglected TBM and a multitude of important questions concerning the diagnosis, pathophysiology and treatment of TBM require answers. Can currently available diagnostic methods be improved? What is the role of molecular diagnostic tests?

What are the mechanisms that lead to coma and death? Do corticosteroids improve outcome? These are some of the major questions that need answering, and this thesis will address all but the last. The answer to this question, in the form of a randomised comparison of adjunctive dexamethasone and placebo in 545 Vietnamese adults with TBM, will be reported elsewhere.

CHAPTER 2

MATERIALS AND METHODS

2.1 Introduction

This chapter describes the general setting of the work undertaken, the region, the hospital and the study wards, and outlines the focus, aims, and structure of the thesis. Laboratory methods are also described, although further relevant details are provided in subsequent chapters.

2.1.1 Geography

Vietnam is the second largest country in South East Asia after Indonesia, and lies between 8° and 24° north of the Equator. It is the largest and most populous of the three Indochinese countries, and borders Cambodia and Laos in the west, and the People's Republic of China in the north. It stretches over 1,600 km along the eastern cost of the Indochinese Peninsula and covers an area of 329,560 square kilometers (**Figure 2.1**).

It is divided into 3 regions:

- Northern Vietnam consisting of provinces bordering China and those that lie in the Red River Delta.
- Central Vietnam with provinces lying between the central coast and the Truong Son mountain range.

- Southern Vietnam which includes Ho Chi Minh City, the few provinces east of the city, and the rice-rich provinces of the Mekong River Delta.

Administratively, Vietnam consists of 53 provinces, divided into 555 districts and further subdivided into 9,960 communes. The capital city, Hanoi, lies within the Red river delta in the north of the country. The research described in this thesis was all undertaken in Ho Chi Minh City, which is situated by the Mekong river delta in the south.

Figure 2.1 Map of Vietnam

Vietnam is home to 79 million people (UK 59 million), the majority of whom live in rural areas. Ho Chi Minh City (formerly Saigon) is the largest city with a population of 7 million. Approximately 40% of the populations are under 15 years old, with 2 million children born each year.

The climate varies throughout the country. It is tropical in the south, with warm and humid weather all year round (22-35°C). The dry season extends from November to April, and it rains between May and October. In the north, temperatures fall from November to April to an average 16^0C, but the summer months are very hot and humid.

2.1.2 Health Care

Vietnam's health care system is positioned 160^{th} in the World Health Organisation's ranking of health care systems. Infant mortality is 34 per 1000 live births and under 5's mortality rate is 45/1000 (UK rates 5 and 7 respectively) (WHO, 2002). The average life expectancy for men is 66.9 years, and 71.8 years for women.

In 2002, Vietnam had a gross national product (GNP) per capita of $390 (UK: GNP $24,420) (WHO, 2002). The health expenditure per capita was $129 /year. Vietnam has a well-developed health infrastructure formed by nearly 10,000 commune health centers, each serving around 8000 people. There are approximately 850 government hospitals employing 27000 doctors. Recently, the private sector has developed rapidly in urban areas, particularly in Ho Chi Minh City, where ATC and other antibiotics are freely available over the counter.

2.1.3 Tuberculosis in Vietnam

In 2001, the estimated incidence of all cases of tuberculosis in Vietnam was 179/100 000 population, which ranked 13th highest in the world. These figures, along with data regarding case detection rates, and directly observed therapy (DOT), are shown in **Table 2.1.**

Table 2.1 Incidence, HIV associated disease, multi-drug resistance, and case detection and treatment of tuberculosis in Vietnam, 2000 (WHO, 2003).

Population	79 174 738
Estimated incidence (all cases/100 000 population)	179
Estimated incidence (new sputum smear +/100 000 population)	80
Estimated % of adult (15-49 years) cases HIV +	1.4
Estimated % new cases multidrug resistant	2.3
Case detection rate (new sputum smear +, %)	84%
DOT treatment success rate (%)	92%

2.1.4 Tuberculosis control in Vietnam

Vietnam has an excellent tuberculosis control program according to government and WHO statistics (WHO, 2003). In 2001, for the 5th consecutive year, Vietnam met both of the global targets for tuberculosis control: 84% of cases were detected, and 92%

treatment success rate was reported. DOT was reported to be available to 100% of the population (WHO, 2003).

However, despite the performance of the control program, the incidence of tuberculosis has not changed over the last 4 years, and there has been no demonstrable impact on the prevalence or death rate from tuberculosis. The reasons for this are likely to be complex, but probably include a developing private sector with inadequate training in tuberculosis treatment and control, poor access to control services for certain high risk groups (drug users, prisoners, illegal residents), an unregulated drug market with use of non-standard anti-tuberculosis drugs, and increasing prevalence of HIV infection.

2.1.5 Hospital for Tropical Diseases

The Hospital for Tropical Diseases (HTD) in Ho Chi Minh City acts as a primary, secondary and tertiary referral centre for patients with infectious diseases, and serves the whole of southern Vietnam (population around 35 million). The hospital has approximately 500 beds, including separate paediatric, adult, and tetanus intensive care units. HTD has laboratories for haematology, biochemistry, microbiology, serology and parasitology. In September 2002 these facilities were moved to a new building, and now share space with the Oxford University research laboratories.

2.1.6 Oxford University Clinical Research Unit

The Oxford University Clinical Research Unit opened in 1991, funded by the Wellcome Trust of Great Britain. The Unit, located within the HTD, serves as a collaborative centre between HTD and Oxford University and started as an 8-bed ward for the treatment of patients with severe malaria. Over 10 years the Unit has expanded and now performs research on five core areas: malaria, dengue, typhoid, central nervous system infection and tetanus.

The Research for this thesis was performed on the Clinical Research Unit (CRU), a 15 bed ward for the treatment of adults (>15 years) with central nervous system infection, severe malaria, severe sepsis and acute renal failure. The CRU has facilities for mechanical ventilation and haemofiltration. Adults with TBM were treated for the first few weeks on this ward, and then transferred to a general recovery ward. Further care and follow-up was coordinated through the staff of this ward. Staff from this ward visited adults failing to attend follow-up visits.

A number of collaborative links exist with other specialist hospitals in the city, in particular Pham Ngoc Thach Hospital for tuberculosis. This hospital coordinates the National Tuberculosis Control program for southern Vietnam, and serves as the secondary and tertiary referral hospital for the region with 500 beds for adults and children suffering from severe tuberculosis.

2.2 Focus, aims and structure of the thesis

This thesis focuses upon the diagnosis and pathophysiology of TBM and aims to address three questions:

1. **What is the best method for distinguishing TBM from other central nervous system disorders?**
2. **How does disease pathophysiology relate to treatment and clinical outcome?**
3. **What other variables influence prognosis?**

Each chapter examines a single hypothesis relating to this question (**Table 2.2**).

Table 2.2. Hypothesis examined by each thesis chapter

CHAPTER	HYPOTHESIS
3	Simple clinical features are predictive of a diagnosis of TBM
4	The sensitivity of conventional bacteriology are dependent upon volume of CSF examined, and duration of microscopy
5	NAA (MTD) is more sensitive than conventional bacteriology for the diagnosis of TBM
6	The cellular and molecular intra-cerebral immune response predicts death or survival from TBM
7	Death or survival from TBM is dependent upon HIV infection, drug resistance, and bacterial genotype

Chapter 8 unites the findings, examines whether the thesis has fulfilled its two aims, and discusses directions for future research.

2.3 Clinical Methods

2.3.1 Scientific and ethical approval

HTD scientific and ethical committee approved all study protocols, and informed consent was obtained from each patient or accompanying relative.

2.3.2 Patients and treatment

The adults described in this thesis were all admitted to the CRU between 1998 and 2003. The timing of the individual studies, and how they relate to the structure of this thesis are presented in **Figure 2.2**. All adults (>15 years old) with a diagnosis of TBM were eligible to enter the studies described.

The diagnostic criteria for TBM was the same for all studies:

1. **Definite TBM:** AFB seen and/or *M.tb* cultured from the CSF
2. **Probable TBM:** clinical signs of meningitis and/or evidence of active tuberculosis infection on chest X-ray and/or AFB isolated from any extra-neural site and/or clinical evidence of extra-pulmonary/extra-neural tuberculosis.
3. **Possible TBM:** clinical signs of meningitis and 4 or more of the following: history of tuberculosis; gradual onset (>5 days); reduced GCS; focal neurology; yellow CSF; CSF lymphocyte predominance; low CSF glucose.
4. **Not TBM:** *either* positive CSF gram stain or culture *or* recovery without ATC

Figure 2.2 Overview of the timing of the studies performed on the CRU for this thesis

Year / Chapter	1998	1999	2000	2001	2002	2003
3	Development and testing of clinical diagnostic algorithm for TBM					
4			The role of ZN stain and culture for the diagnosis of TBM			
5			The role of MTD for the diagnosis of TBM			
6			Pathophysiology of TBM			
7	Prognostic impact of HIV infection, drug resistance, and mycobacterial genotype					

Severity of TBM at the start of treatment was graded according to a modified MRC criteria (MRC, 1948b): Grade I had a Glasgow coma score (GCS) of 15/15 with no focal neurological signs, grade II either had a GCS 11-14 or GCS 15 with focal neurological signs, Grade III had a GCS of ≤ 10.

All patients with TBM received daily intra-muscular streptomycin 20mg/kg (maximum 1g), and oral isoniazid 5 mg/kg, rifampicin 10mg/kg, and pyrazinamide 30mg/kg for 3 months, followed by 3 drugs (isoniazid, rifampicin, pyrazinamide) for 6 months. Oral ethambutol was substituted for streptomycin in those with HIV infection to reduce the needle-stick injury risk to staff and because of the high prevalence of streptomycin resistant *M.tb* in this population.

None of the patients described received corticosteroids, although a large randomised controlled trial is being performed to address this issue. Clinical data were recorded prospectively in individual study notes, and entered onto a Microsoft excel worksheet. All the data were double-checked before analysis.

2.3.3 Routine investigations

It is routine clinical practice in HTD for all patients with TBM to have a lumbar puncture at diagnosis (day 0), day 3, day 7, day 30, day 60, and day 270 of treatment. Like others (Feigin RD *et al.*, 1973; Smith HV, 1963; Teoh R *et al.*, 1986), repeated lumbar punctures are considered helpful in management, particularly when assessing poor

response to ATC or when the diagnosis remains questionable. Computerised tomography (CT) of the brain before lumbar puncture was arranged in all patients with a clinical suspicion of raised intra-cranial pressure (coma and/or focal neurological signs).

CSF cell counts and biochemistry were performed by standard methods upon each sample. CSF lactate was only measured during the first 30 days of treatment. Routine microbiological investigation included microscopy of the centrifuged CSF deposit after Ziehl-Neelsen, Gram's and Indian ink stains, with culture for fungi, pyogenic bacteria, and mycobacteria. CSF supernatant and deposit from each sample submitted for microbiological analysis was stored at -70^{0}C. Serum and plasma taken at the time of lumbar puncture was frozen at -70^{0}C.

2.4 Laboratory methods

2.4.1 Bacteriological diagnosis of tuberculous meningitis

Preparation of specimens

Taking large volumes of CSF probably improves bacteriological yield (Kennedy DH *et al.*, 1979; Stewart SM, 1953), therefore the CRU physicians collected 5-10mls into a 30ml clear sterile universal container from all patients with suspected TBM. In each case the volume of CSF was recorded and centrifuged at 3000 x g for 15 minutes as soon as possible after collection.

After centrifugation most of the supernatant was removed using a sterile pipette, and stored in eppendorfs at -70^{0}C. These frozen samples were used later for the experiments on pathophysiology (**Chapter 6**).

The deposit was vigorously re-suspended in the remaining 300-400µl of supernatant. Two drops (approximately 100µl each drop) were dried onto a heated clean slide (diameter <1cm), two drops were frozen at -70^{0}C for later testing by *Mycobacterium Tuberculosis* Direct (MTD) test (Gen probe, USA) (**Chapter 5**), and remaining deposit was divided equally between for aerobic culture on Lowenstein-Jensen media and liquid Mycobacterium Growth Indicator Tubes (MGIT, Becton Dickinson, USA).

Ziehl-Neelsen stain of CSF

The reagents for the ZN method were prepared according to Collins et al (Collins CH *et al.*, 1997).

i) *Preparation of the ZN stain:*

5g basic fuchsin

25g phenol crystals

50mls ethanol, 95%

500mls distilled water

The fuchsin and phenol were dissolved in the ethanol, and then the water added.

The solution was filtered before use.

ii) *Preparation of the acidified alcohol decolourising solution*

970mls ethanol, 95%

30mls concentrated hydrochloric acid

Mixed well.

iii) *Preparation of the Counter-stain*

2.5g methylene blue

500mls distilled water.

Mixed well.

Once the drops of deposit had dried, the slide was passed through a Bunsen flame twice in quick succession and placed on a staining rack over a sink. Filtered ZN stain was poured over the slide until the deposit was well covered. The slide was then heated from below with a spirit lamp flame until steam rose from the stain. The stain was not allowed to boil, or dry on the slide.

After 5 minutes the slide was washed well with running water, and covered with acid-alcohol for 3-5 minutes, or until all the stain had left the deposit. After careful washing with running water the slide was placed on a heating block for 1-2 minutes. Without this step the deposit was frequently washed off the slide when the methylene blue was added. Once the deposit was dry, it was covered with methylene blue for 30-60 seconds, and washed very carefully with running water.

Once dry, the slide was first examined under low power for areas of high cellular density. Experience showed that AFB were most frequently found in these areas; therefore they were examined first under high power using an oil-emersion lens. If these areas proved negative, the rest of the slide was systematically examined for at least 20 minutes. A slide was termed positive if 2 or more bacilli were seen, and the time to see the bacilli was recorded. The time spent looking at negative slides was also recorded.

Culture, identification, and drug susceptibility testing of M.tb

CSF cultures were examined weekly for signs of growth for 12 weeks. Once the presence of AFB was confirmed by a ZN stain, cultures were sent the Pham Ngoc Thach Hospital laboratory for identification and susceptibility testing. This laboratory serves as the tuberculosis reference laboratory for southern Vietnam, and their performance is audited by the WHO quality control scheme for mycobacterial identification and drug susceptibility testing. Briefly, *M.tb* was identified on the basis of acid-fastness, and 4 biochemical tests:

i) A positive Niacin test

ii) Positive Nitratase activity

iii) Inhibition of growth by 500mg/l *p*-nitrobenzoic acid (PNB)

iv) Growth in the presence of thiopen-2-carboxylic acid hydrazine (TCH)

Drug susceptibility was performed using the proportion method (Collins CH *et al.*, 1997). In principle, the number of colonies growing on drug-free medium is compared with the

number on drug-containing medium and the proportion of resistant organisms is calculated. Pham Ngoc Thach laboratory compared growth on drug free media, with growth on media with the following concentration of drugs:

Pyrazinamide 200 μg/ml

Isoniazid 0.2 μg/ml

Streptomycin 4 μg/ml

Rifampicin 40 μg/ml

Ethambutol 2 μg/ml

The criterion for resistance was the number of colonies on the drug-containing medium was 1% or more of the number developing on the drug-free medium.

2.4.2 Amplified Mycobacterium Tuberculosis Direct Test

All reagents and equipment were purchased from Gen-Probe (California, USA) at commercial rates. The manufacturer's instructions were followed for all steps except for the pre-treatment of CSF, where a modified protocol was used.

Preparation of specimens and lysis

The MTD was developed for use on respiratory samples and is not licensed for use with CSF. Previous authors have suggested alterations to the manufacturers protocol may improve performance with CSF (Pfyffer GE *et al.*, 1996). In particular, these authors found increased sensitivity by increasing the volume of sample, pretreatment with a

denaturing agent, and increasing the amplification time from 2 to 3 hours. Further details of this method are provided in **Chapter 5**.

Amplification and hybridisation

1. 25 µl of lysate was added to the bottom of an amplification tube containing 50 µl of reconstituted amplification reagent and 200 µl oil reagent.
2. Incubated for 15 minutes at 95^0C.
3. Tube transferred to 42^0C and allowed to cool for 5 minutes
4. 25 µl of reconstituted enzyme reagent added, mixed, and incubated for 3 hours
5. 100 µl of reconstituted hybridisation reagent added to tube, vortexed, and incubated at 60^0C for 15 minutes.
6. 300 µl of selection reagent added to tube, vortexed, and incubated at 60^0C for 15 minutes. Then cooled to room temperature.
7. Insert tube into luminometer to obtain RLU readout

Controls

H37Rv *M.tb* was used as the positive controls and *Mycobacterium avium* as the negative control.

2.4.3 Cytokine and matrix metalloproteinase assays

Commercial capture ELISA kits were used to measure CSF and blood concentrations of IFN-γ, TNF-α, IL-8, and IL-10 (OPTEIA ELISA, Becton Dickinson, San Jose,

California, USA), and the CSF concentrations of MMP-9, TIMP-1, and the soluble TNF-α receptors 1 and 2 (R and D systems, Abingdon, UK). The manufacturers methods were followed in each case. The lower limits of detection were 10 pg/ml of IFN-γ, TNF-α, TNF-α R1, TNF-α R2, and IL-8; 15 pg/ml of IL-10; 200 pg/ml of TIMP-1, and 350 pg/ml of MMP-9.

2.4.4 Albumin and IgG indices

A commercial analyser measured albumin and IgG concentrations in blood and CSF (Hitachi 917, Hitachi, Japan) by immunoturbimetry using polyclonal anti-human albumin, or IgG antibodies (Roche diagnostics, Switzerland). The albumin index (AI) was calculated by $[albumin]_{csf}/[albumin]_{plasma}$. Normal ranges for albumin in plasma are 36-50 g/l, and 0.16-0.36 g/l in CSF (Fishman R, 1992): the normal range for AI is 0.0032- 0.01. The formula used to calculate the IgG index (IgGI) was $([IgG]_{csf} \times [albumin]_{plasma})/([albumin]_{csf} \times [IgG]_{plasma})$ (Fishman R, 1992). The normal range for the IgG index is 0.34-0.58 (Fishman R, 1992).

CHAPTER 3

THE CLINICAL DIAGNOSIS OF TUBERCULOUS MENINGITIS

3.1 Introduction

The diagnosis of TBM is dependent upon isolating *M.tb* from the CSF, but culture is too slow to aid clinical decision-making. Direct ZN staining of the CSF for AFB remains the cornerstone of rapid diagnosis in most laboratories, but the sensitivity is variable. Newer diagnostic techniques, such as those employing the polymerase chain reaction (PCR), are incompletely evaluated (Pai M *et al.*, 2003) (Pfyffer GE, 1999) and not currently possible in most settings in the developing world where the majority of TBM is seen (Foulds J *et al.*, 1998; Wilson SM, 1998). As a consequence the decision to treat a patient for TBM is frequently empirical, regardless of the diagnostic facilities available to the clinician, and the clinician must depend upon the discriminative clinical and laboratory features of the disease for successful diagnosis and treatment.

The presenting clinical features of TBM in adults are similar to many meningo-encephalitides and results in frequent diagnostic confusion. Delay in starting appropriate antibiotics for TBM or pyogenic meningitis worsens prognosis, yet physicians are often reluctant to start months of anti-tuberculosis treatment without firm evidence.

Diagnostic uncertainty arises commonly in patients presenting with a few days of headache, fever and neck-stiffness; undefined treatment in the community; a low CSF glucose (<50% of blood), and neutrophils and lymphocytes in the CSF.

As described in **Chapter 1**, multivariate logistic regression has been used to model the clinical predictors of TBM in 232 children (Kumar R *et al.*, 1999). Five presenting clinical features were found to be independently predictive of the diagnosis of TBM: prodromal stage ≥ 7 days, optic atrophy on fundal examination, focal deficit, abnormal movements, and CSF leucocytes < 50% polymorphs. The authors developed a simple diagnostic rule: sensitivity was 98%, and specificity 44%, when one or more predictor variables were present; and specificity was 98% and sensitivity 55%, if three or more were present. Although this, and similar rules have their limitations (see **Chapter 1**), there are strong arguments for developing a simple diagnostic algorithm for TBM in areas of high tuberculosis prevalence. First, TBM tends to be commonest in areas with the least clinical and laboratory resources. Second, a diagnostic rule developed and used in high tuberculosis prevalence areas may perform consistently. And third, early diagnosis and treatment improves outcome (Kennedy DH *et al.*, 1979). This chapter examines the hypothesis that simple clinical features are predictive of a diagnosis of TBM.

3.2 Methods

The adults described in this study were admitted to the CRU as described in **Chapter 2.** An HIV test was not performed on all patients in this study. In general, only those considered to be at risk of infection were tested. A second lumbar puncture was performed as part of routine hospital management in most patients after 48 hours to assess response to treatment. Those with culture proven or suspected bacterial meningitis (BM) received 10 days of intra-venous ceftriaxone (Rocephin®, Roche) (2 grams twice a day). Those with TBM were treated with ATC as described in **Chapter 2.**

Table 3.1 Diagnostic criteria for TBM and BM.

Tuberculous Meningitis	**Bacterial meningitis**
M.tb isolated from the CSF	Pathogenic bacteria isolated from the CSF
Or	***Or***
Clinical meningitis with negative Gram and India ink stains, and sterile bacterial and fungal cultures, and one or more of the following:	Clinical meningitis with all of the following:
1.Cranial CT consistent with TBM (hydrocephalus, oedema, basal meningeal enhancement)	1. Lymphocytes and neutrophils in the CSF
2. Chest radiograph consistent with active pulmonary TB	2. Low CSF glucose (<50% blood)
3. Good response to ATC.	3. Sterile blood and CSF cultures.
	4. Full recovery (without ATC) 3 months after admission

3.2.1 Diagnostic criteria

Diagnostic criteria for TBM and BM were applied to all patients admitted to the CRU with meningitis. The criteria are presented in **Table 3.1.** There is no acceptable 'gold standard' diagnostic test for TBM. Culture and ZN staining of the CSF are specific, but too insensitive to be used as the sole criteria for diagnosis. Clinical criteria were therefore developed for this study that are comparable to those used by other authors (Ahuja GK *et al.*, 1994). The criteria for the diagnosis of BM aimed to include partially treated BM (with sterile CSF cultures) in the study. This group is important as diagnostic confusion with TBM occurs commonly in these patients. TBM was excluded by their full recovery, without ATC, 3 months after admission: untreated TBM would almost always be fatal within this time.

3.2.2 Statistical methods

The clinical and laboratory features of those fulfilling the diagnostic criteria for TBM and BM were compared. Twenty-six admission clinical and laboratory parameters, including characteristics of the admission CSF sample, were studied. Additionally, data from a second CSF sample were collected from all patients receiving 48 hours of intra-venous ceftriaxone. Diagnostic uncertainty on admission often leads clinicians in our hospital to use a 'trial' of ceftriaxone, and reconsider the diagnosis after a 2nd CSF examination. The purpose of collecting these additional data was to compare the CSF parameters in both groups, and to develop a second diagnostic rule for patients managed by this approach.

The relative change in each CSF parameter was calculated by (a(CSF 2) – a(CSF 1))/a(CSF 1), where a is the specified parameter. All those given immediate ATC were excluded from this analysis. Kruskal-Wallis test was used to compare continuous parameters; the chi square test (or Fisher's exact test for small proportions) was used for categorical variables.

Three diagnostic aids were developed using two statistical approaches: classification trees (CART), and logistic regression. The classification trees were developed by consideration of all the variables separately. The range of each variable was divided into two groups to obtain the best separation between the BM and TBM patients. The division corresponding to the best separation was selected. The resulting subsets of cases were then partitioned independently in turn. The process was performed recursively, until a stopping condition was satisfied. Node deviance, which measures node heterogeneity, was set to 0.1 to stop the tree growing process. Subsets smaller than 10 were not partitioned further.

Logistic regression was used to model the probability of having TBM. A stepwise forward variable selection procedure was employed to find independent predictors of TBM with p-to-enter ≤ 0.05, and p-to-remove ≥ 0.055. Once the final model was constructed, the continuous variables in the model were dichotomized using cutoffs from the univariate classification trees, and the model was refitted. Rounded β-coefficients from the model with dichotomized variables were used to define a diagnostic index (DI) for each of the clinical variables. A receiver operator characteristic (ROC) curve analysis

was selected to find an optimal cutoff for the combined diagnostic indices. ROC analysis was performed upon the original dataset, and the completed rule (DI with cutoff) was applied to the test data.

The three diagnostic aids were evaluated using resubstitution and prospective test data methods. The sensitivity and specificity were calculated and compared with the study diagnostic criteria. The resubstitution method used the original data set. The test data method employed data recorded from a further 75 patients enrolled in the same manner and subject to the same diagnostic criteria. All analyses were performed using STATA and Splus.

3.3 Results

3.3.1 Adults satisfying diagnostic criteria

Three hundred and fifty-seven adults were admitted to the CRU with meningitis between 1998-2000. 251 satisfied the diagnostic criteria for inclusion in this study: 143 with TBM, and 108 with BM. An HIV test was performed in 66/251 adults: 8 were positive (7 had TBM, 1 had BM).

163/357 adults received ATC for suspected TBM: *M.tb* was isolated from the CSF of 37 patients, and 106 were defined as having clinical TBM. Supportive radiological evidence for TBM was present in 85/106 adults with clinical TBM. 20 patients treated for TBM were excluded because they did not meet the study criteria for the diagnosis of TBM, and all died shortly after the start of ATC. No significant ($p<0.05$) differences were found

between the admission clinical and laboratory parameters of those with culture-confirmed and clinical TBM.

194/357 adults admitted over the same period were not treated for TBM: 108 satisfied the study criteria for a diagnosis of BM. A bacterial pathogen was isolated from the CSF of 68 adults (**Table 3.2**). A further 40 adults satisfied the diagnostic criteria for BM (presumed partially treated). 86 adults were excluded from the study for the following reasons: 61 had a normal CSF glucose (>50% blood), 15 died within 3 months, and 11 were lost to follow-up. Admission variables are shown in **Table 3.3.**

Table 3.2 Microbiology of culture proven bacterial meningitis.

Organism from CSF	**Number**	**Percentage**
Streptococcus pneumoniae	24/68	35.3%
Streptococcus suis	31/68	45.6%
Neisseria meningitidis	4/68	5.9%
Haemophilus influenzae	4/68	5.9%
Klebsiella sp	4/68	5.9%
Escherichia coli	1/68	1.5%

Table 3.3 Univariate analysis comparing admission parameters between patients with TBM and BM

Parameter	Tuberculous meningitis			Bacterial meningitis			P-value
	Median	90% range	(n)	Median	90% range	(n)	
Age (years)	34	16-64	(143)	41	17-70	(108)	0.008
Male sex	91 (64%)			84 (78%)			0.016
Duration of illness (days)	12	4-34	(142)	3	1-11	(107)	<0.001
Duration of fever (days)	10	2-30	(139)	3	1-11	(106)	<0.001
Duration of headache (days)	10	1-30	(136)	3	1-11	(106)	<0.001
Neck stiffness	120 (91%)		(143)	81 (84%)		(106)	0.091
Presence of coma before admission (Yes)	49 (36%)		(143)	53 (50%)		(106)	0.027
Glasgow coma score (/15)	13	7-15	(143)	14	6-15	(107)	0.091
Pulse (/min)	90	60-120	(143)	92	72-120	(108)	0.003
Systolic BP (mmHg)	120	90-150	(143)	120	90-150	(108)	0.76
Diastolic BP (mmHg)	70	50-90	(143)	70	50-90	(108)	0.24
Hemiplegia	11 (8%)			4 (4%)			0.28
Cranial nerve palsies	32 (22%)		(143)	9 (8%)		(107)	0.003
Haematocrit (%)	40	30-48	(139)	42	30-48	(104)	0.12
White blood cell count (10^3 /ml)	9800	5000-16200	(137)	15250	7470-31500	(107)	<0.001
% neutrophils	80	60 – 89	(130)	86	70 – 95	(107)	<0.001
Blood sodium (mmol/l)	135	122-143	(98)	138	125-148	(102)	0.001
CSF opening pressure (cm H_2O)	23	9-44	(133)	24	7.5-40	(60)	0.92
Clear CSF appearance	81(57%)		(141)	2 (2%)		(107)	<0.001
CSF total WCC (10^3 /ml)	300	70-1090	(143)	2583	382-20000	(108)	<0.001
CSF % neutrophils	37	1-84	(142)	90	60-99	(108)	<0.001
CSF % lymphocytes	64	16-99	(142)	10	1-40	(108)	<0.001
CSF/Blood glucose	0.28	0.11-0.52	(139)	0.20	0.03-0.46	(101)	<0.001
CSF chloride (mmol/l)	108	85-120	(136)	109	97-121	(67)	0.012
CSF protein (g/dl)	191	80-490	(141)	270	89-730	(107)	<0.001
CSF lactate (mmol/l)	5.4	1.5-9.8	(102)	9.4	2.1-19.7	(92)	<0.001

Excluding the 92 patients who received immediate ATC, the results from a 2nd lumbar puncture performed after 48 hours of ceftriaxone were available in 157/251 patients - 51 patients with TBM, and 106 patients with BM (**Table 3.4**).

Table 3.4 Second CSF analysis taken after 48-72 hours of parenteral ceftriaxone.

	TBM			BM			
48hr CSF	Median	90% range	(number)	Median	90% range	(number)	**p-value**
CSF opening pressure (cm H_2O)	27	8-44	(43)	14	4-27	(58)	0.003
Clear CSF appearance	21 (49%)			23 (23%)			<0.001
CSF total WCC (10^3 /ml)	470	173-2575	(51)	760	80-12000	(106)	0.048
CSF % neutrophils	47	4-87	(51)	74	8-97	(106)	<0.001
CSF % lymphocyte	53	13-96	(51)	26	3-90	(106)	<0.001
CSF/Blood glucose	0.27	0.13-0.5	(47)	0.43	0.11-0.69	106)	<0.001
CSF chloride (mmol/l)	102	81-116	(48)	115	105-126	(64)	<0.001
CSF protein (g/dl)	190	98-680	(49)	110	37-580	(104)	<0.001
CSF lactate (mmol/l)	6.4	2.9-11	(36)	4.8	1.8-10.4	(77)	0.011

2/251 patients with culture confirmed BM died before a 2nd sample could be taken. After 48 hours of ceftriaxone a low CSF glucose (<50% blood) was found in 68/106(64%) of adults with BM, and 44/51(94%) of those with TBM (**Table 3.5**)

Table 3.5 Change in the CSF parameters after 48-72 hours of parenteral ceftriaxone as calculated by (CSF2 – CSF 1/ CSF 1). Expressed as % change.

	TBM Median % change 90% range number	**BM** Median % change 90% range number	**p-value**
CSF opening pressure	+8% –75 to +267% 36	-41% -83 to +56% 51	<0.001
CSF WCC	+13% -68 to + 964% 51	-75% -97 to + 918% 106	<0.001
CSF % Neutrophils	-1% -45 to + 500% 50	-15% -88 to + 23% 106	0.001
CSF % lymphocytes	+5% -54 to +145% 50	+144% -88 to +155% 106	<0.001
CSF glucose/blood ratio	-6% -66 to + 92% 45	+100% -61 to + 833% 99	<0.001
CSF lactate	+0% -55 to + 314% 27	-48% -81 to + 114% 70	<0.001
CSF Protein	+2% -37 to + 275% 49	-54% -90 to + 51% 103	<0.001
CSF chloride	-4% -14% to +8% 48	+4% -8 to +21% 63	<0.001

3.3.2 Diagnostic classification trees

Figures 3.1 and 3.2 show the diagnostic trees generated from the CART analysis. **Figure 3.1** is for use on admission; **Figure 3.2** is for use after 48 hours of broad-spectrum antibiotics.

Figure 3.1 Admission diagnostic classification tree

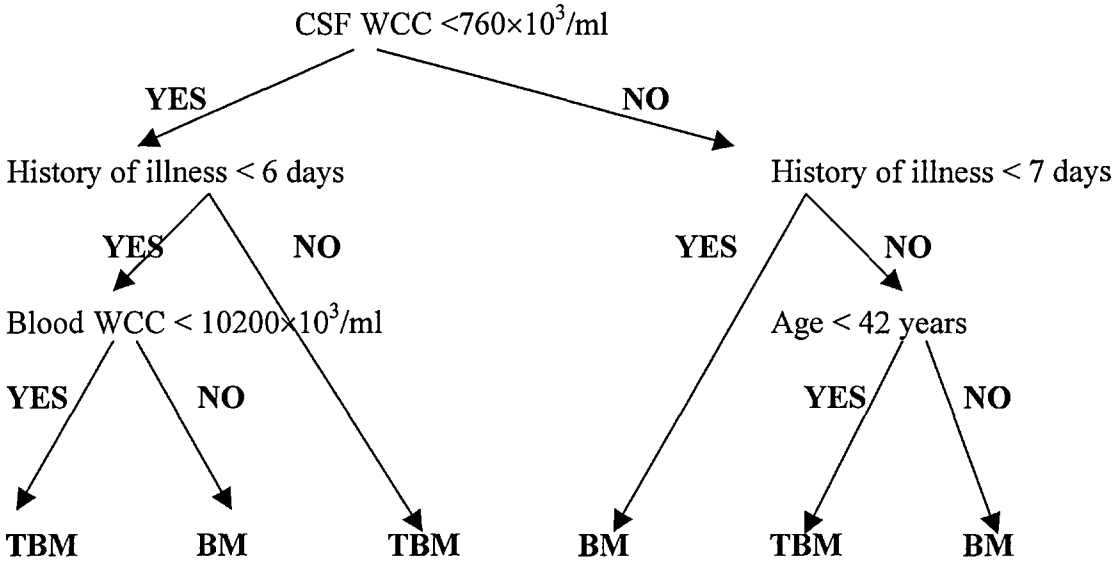
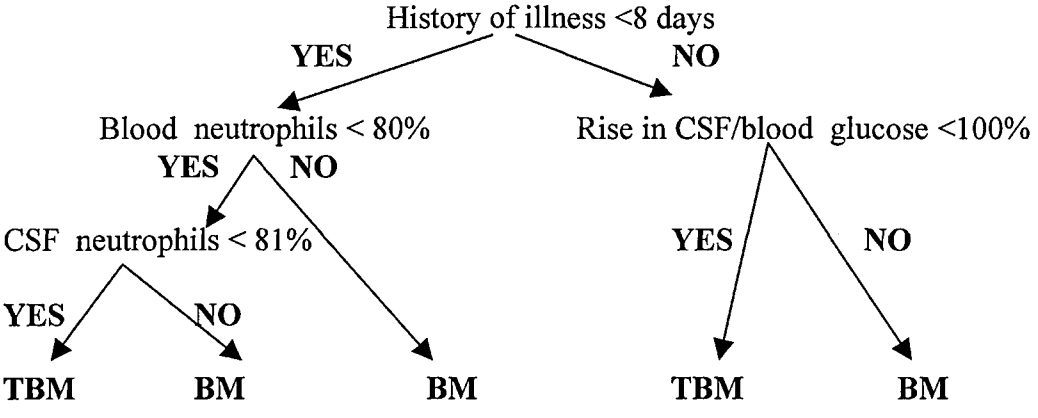


Figure 3.2 Diagnostic classification tree for use after a trial of 48 hours broad spectrum antibiotics



3.3.3 Diagnostic rule from Logistic regression

Multivariate analyses were performed to construct a diagnostic rule. A number of variables were excluded from the analyses. Admission Glasgow Coma Score, diastolic and systolic BP, presence of hemiplegia, and blood haematocrit, were excluded due to the non-significant ($p>0.05$) results by univariate analysis. Blood sodium, CSF opening pressure, CSF chloride, and CSF lactate were excluded because of a large number of missing values. Only the total duration of combined symptoms before admission was included for analysis: the length of fever, and headache, were excluded.

Stepwise logistic regression analysis found 5 variables independently associated with a diagnosis of TBM on admission. The final logistic model with these variables is described in **Table 3.6.**

Table 3.6 Multivariate logistic regression analysis of admission data

	β-Coefficient	**OR**	**(95% CI)**	**p-value**
Age	-0.069	0.933	(0.885-0.984)	0.010
History of illness	0.243	1.275	(1.132-1.437)	<0.001
Blood WCC	-0.0003	0.9997	(0.9995-0.9999)	0.001
CSF WCC	-0.002	0.998	(0.996-0.999)	<0.001
CSF % neutrophils	-0.075	0.928	(0.884-0.973)	0.002

The formula for diagnostic index (DI) was derived from the final model by dichotomizing the variables and rounding the coefficients in the model. The index was adjusted to a positive scale for ease of use.

The DI for each of the 5 variables is given in **Table 3.7**

Table 3.7 Weighted Diagnostic index scores for the dichotomized clinical variables used for the admission diagnostic rule

Variable	**DI**
Age (years)	
≥ 36	+2
< 36	0
Blood WCC (10^3/ml)	
≥15000	+4
<15000	0
History of illness (days)	
≥ 6	-5
< 6	0
CSF total WCC (10^3/ml)	
≥ 900	+3
< 900	0
CSF % neutrophils	
≥ 75	+4
< 75	0

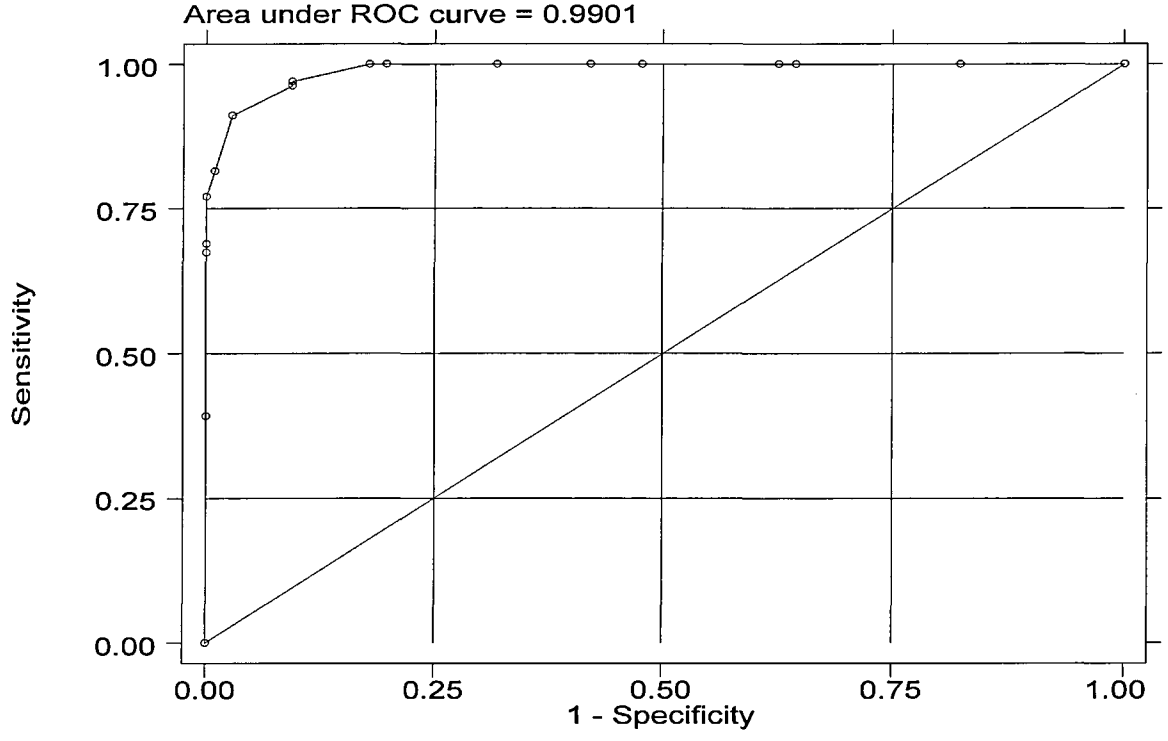
Suggested Diagnostic Rule: Total DI score ≤ +4 = TBM

Total DI score > +4 = BM

The **Total DI** was calculated for each patient according to the formula:

Total DI = DI (age) + DI (blood WCC) + DI (history of illness) + DI (CSF WCC) + DI (CSF % neutrophils). The optimal cut-off for **Total DI** (to classify a patient as a TBM case) was found using a ROC curve (**Figure 3.3**). There are three points close to the top left-hand corner of the curve: these correspond to cut-offs of +4, +3, or +2. The respective diagnostic sensitivities and specificities for each point are 97%, 91%; 96%, 91%; and 91%, 97%.

Figure 3.3 Receiver-operator characteristic curve for prognostic index derived from the logistic regression model.



No improvement in the diagnostic rule was obtained by employing data from both admission and the second CSF. Missing values, which reduced the sample size to 121 observations, may have accounted for this failure.

Table 3.8 Misclassification rates of the three diagnostic aids

	Resubstitution method		***Test data method***	
	Sensitivity	**Specificity**	**Sensitivity**	**Specificity**
At admission:				
Classification tree	99% (134/136)	93% (99/107)	88% (37/42)	70% (23/33)
Diagnostic rule	91% (123/135)	97% (104/107)	86% (36/42)	79% (26/33)
After 2nd CSF:				
Classification tree	93% (39/42)	95% (92/97)	57% (8/14)	76% (25/33)

3.3.4 Performance

The performances of each of the diagnostic aids (from CART or logistic regression) are summarized in **Table 3.8**. Resubstitution of the original data set into the admission diagnostic tree misclassified 10 patients (2 TBM and 8 BM) giving 99% sensitivity and 93% specificity.

The second tree misclassified 8 patients (3 TBM and 5 BM) giving 93% sensitivity and 95% specificity.

The test data were recorded from a further 75 patients satisfying the study diagnostic criteria: 20 adults had culture-confirmed TBM; 22 adults had clinical TBM; 21 adults had culture-confirmed BM; and 12 adults had clinical BM. Clinical data from this group was applied to each diagnostic aid: the admission tree (**figure 3.1**) was 88% sensitive and 70% specific; the admission diagnostic rule (**table 3.7**) was 86% sensitive and 79% specific; and the second diagnostic tree (**figure 3.2**) was 57% sensitive and 76% specific.

3.4 Discussion

The diagnosis of TBM in adults is difficult regardless of the resources available to the physician. This study employed diagnostic criteria that derive from CSF culture results, and observed response to specific treatment. Such criteria suggest that even in the best settings a proportion of those with TBM or BM will never be proven microbiologically.

As untreated TBM is always fatal it is essential that a clinical diagnostic aid or laboratory assay for TBM is sensitive. The potential toxicity and duration of ATC, and the limited resources of many tuberculosis control programs, also mandates diagnostic specificity. At present no rapid laboratory method for the diagnosis of TBM satisfies these requirements. A clinical diagnostic rule or classification tree for TBM may improve upon the sensitivity of current laboratory methods, and it may be

used in settings with limited microbiological diagnostic support, i.e. where TBM is most common. This study compared patients with TBM with confirmed or probable BM for two reasons: first, both groups of patients require immediate decisions regarding chemotherapy, and second, those with BM, particularly partially treated BM, are difficult to distinguish from those with TBM. Low CSF glucose is usually present in both conditions, and forms an important discriminating feature from other meningo-encephalitides. The CSF:blood glucose ratio is usually >50% in patients with viral meningo-encephalitis. A low CSF glucose (<50% blood) was present on admission in all but one patient entering this study.

The most common agent isolated from patients with BM was *Streptococcus suis*, which has been well described in south east Asia (Kay R *et al.*, 1995). The infection is most commonly seen in men working in frequent contact with pigs, which explains the 78% male preponderance in the BM group. The organisms isolated were all sensitive to ceftriaxone, as were all the isolates of *Streptococcus pneumoniae*. The clinical features of *Streptococcus suis* meningitis are comparable to other bacterial meningitides, and their inclusion in this study is unlikely to affect the application of the diagnostic aids produced. The prevalence of HIV infection in the study population was low, although only 66/251 patients were tested. At the time of the study the prevalence of HIV infection was high in two groups: commercial sex workers, and intra-venous drug addicts (Nguyen TH *et al.*, 1999). The prevalence of HIV infection amongst women attending maternity clinics in Vietnam, 1994-1998 was less than 0.15%.

Although HIV testing was limited to at-risk groups, the study is unlikely to have included substantial numbers of patients with unrecognised HIV infection. Although HIV appears to increase risk of developing TBM (Berenguer J *et al.*, 1992), it may not alter the clinical and laboratory features of the disease (Yechoor VK *et al.*, 1996). However, HIV infection alters the differential diagnosis in meningitic adults: opportunistic infection with unusual pathogens must be considered, in particular *Cryptococcus neoformans*, which may present sub-acutely, similar to TBM. The results of this study should therefore be applied with caution in areas with a high prevalence of HIV infection.

The univariate analysis of admission variables suggests a set of potentially discriminative clinical features. Patients with TBM present with a longer history; they are more likely to have cranial nerve palsies; they will not usually have a blood leucocytosis; and their CSF will frequently be clear, with moderate numbers of lymphocytes and neutrophils, in combination with an elevated protein and a low CSF:blood glucose ratio. However, diagnostic uncertainty frequently persists despite the first CSF analysis. In such cases, physicians in our hospital may assess response to treatment after 48 hours with a broad-spectrum antibiotic. **Tables 3.4 and 3.5** demonstrate significant differences are apparent between the two groups. As expected, the CSF parameters change little in those with TBM. The changes observed in BM presumably reflect successful antimicrobial effect: CSF pressure, white cell count, protein and lactate fall whilst CSF:blood glucose ratio rises.

The dangers of delayed ATC focus attention on a diagnostic aid using admission clinical features. Multivariate logistic regression analysis defines five characteristics independently predictive of a diagnosis of TBM from BM: age, history of illness, blood white cell count, CSF white cell count, and the CSF neutrophil percentage (**table 3.6**). A diagnostic rule can be suggested from the ROC analysis: those patients presenting with a total DI score of +4 or less have TBM, those with a score greater than +4 have BM. A cut-off of +4 was chosen as it provided the greatest sensitivity (97%) with acceptable specificity (91%). Applying the test data the rule is 86% sensitive, and 79% specific, figures that are comparable to the best available laboratory assays (Thwaites G *et al.*, 2000a).

Two diagnostic classification trees were developed: the first for admission, and a second to be used after a trial of broad-spectrum antibiotics (**figures 3.1 and 3.2**). When the test data are applied to the admission tree diagnostic sensitivity is similar to that of the rule (88%), but specificity is reduced (70%). The second tree performs less well, although the data available to test this tree were small: 14 patients with TBM, 33 with BM. Nevertheless, a diagnostic tree incorporating the second CSF is attractive given the striking differences documented between those with TBM and treated BM, but requires further development and prospective assessment.

There are some important limitations to this study. First, the prevalence of both tuberculosis and HIV infection will affect the performance of the rule. Therefore, this

rule should not be used in areas of substantially different tuberculosis and HIV prevalence to southern Vietnam without prospective evaluation in those settings.

As this will remain a fundamental problem for all similar diagnostic methods, future research should also be directed at investigating their impact upon outcome. Early treatment of TBM is known to save lives (Kennedy DH *et al.*, 1979), the impact of a diagnostic rule upon outcome should be prospectively evaluated.

This study suggests that simple clinical and laboratory data can be used to help diagnose TBM in adults, and develops an admission diagnostic rule with 86% sensitivity and 79% specificity. The rule should be applied to adults in high tuberculosis prevalence settings with meningitis and a CSF:blood glucose < 50%. As the diagnosis and management of meningitis rests on clinical and CSF assessment, efforts are necessary to support clinical and appropriate laboratory diagnostic services in low-income countries.

CHAPTER 4

THE BACTERIOLOGICAL DIAGNOSIS OF TUBERCULOUS MENINGITIS

4.1 Introduction

The demonstration of AFB in the CSF by the ZN stain remains the most widely available rapid diagnostic test for TBM. However, there is large and unexplained variability in the sensitivity of this method. Recent authors report bacilli are seen in the CSF of less than 10% of cases (Garg RK, 1999), but the older literature suggests much better results can be achieved. In 1953 Stewart found AFB in the CSF of 91/100 consecutive cases of TBM, all were subsequently confirmed by culture (Stewart SM, 1953). Similar results were reported in 1979 by Kennedy, who found AFB in the CSF of 45/52 (87%) patients treated for TBM (Kennedy DH *et al.*, 1979). The reasons why many laboratories fail to replicate these results are uncertain. Stewart suggested diagnostic performance was dependent upon the volume of CSF submitted for examination, the speed and duration of centrifugation, and meticulous microscopy (Stewart SM, 1953). Kennedy ascribed their results to the repeated and thorough examination of CSF for AFB, even after ATC had

started (Kennedy DH *et al.*, 1979). However, there are few data to support these assertions.

The aim of this study was to prospectively assess the procedural and clinical variables affecting the sensitivity of CSF ZN stain and culture for *M.tb*, and test the hypothesis that the sensitivity of conventional bacteriology is dependent upon the volume of CSF examined and the duration of microscopy.

4.2 Methods

4.2.1 Clinical methods

A special laboratory diagnostic service for TBM was set up at the CRU in 2000, and the attending physicians were encouraged to submit 5-10mls of CSF from any adult admitted with possible TBM. The volume of each CSF sample submitted to the service was recorded, and processed according to the methods described in **Chapter 2**. If two or more AFB were identified the slide was termed positive, and the time to see 2 bacilli recorded. The time taken to examine negative slides was also recorded. Only one slide was made per CSF specimen. The remaining deposit was cultured in liquid Mycobacterial Growth Indicator Tubes (MGIT, Becton Dickenson, USA), and on Lowenstein-Jenson (LJ) media for a minimum of 8 weeks.

The clinical and laboratory features, and the final diagnosis of each patient admitted to the CRU were recorded prospectively in individual study notes as described in **Chapter 2**. All patients with TBM were tested for antibodies to HIV.

4.2.2 Statistical methods

Normally distributed continuous variables were compared by the Student t-test; all other continuous variables were compared by the Mann-Whitney U test. Categorical variables were compared by the chi-squared test. Multivariate analysis by forward logistic regression was performed to model the likelihood of seeing bacilli or isolating *M.tb* from the CSF. The following variables were selected *a priori* to enter the model: age, sex, HIV status, duration of symptoms and disease grade; volume of CSF, CSF opening pressure, CSF total white cell count with neutrophil and lymphocyte percentages, CSF total protein, lactate, chloride, and CSF:blood ratio. The analysis was performed using 'Statistical Product and Service Solutions' (SPSS) software version 10.0 (Microsoft, USA).

4.3 Results

4.3.1 Patients satisfying diagnostic criteria

Seven hundred and twenty CSF specimens from 320 adults were submitted to the laboratory between May 2000 and April 2003: 384 were 'diagnostic' specimens, taken before the start of ATC from 320 adults; 346 samples were taken from 132 adults after starting treatment for TBM.

198 adults (241 specimens) were not treated or diagnosed as having TBM: 70 (35%) had bacterial meningitis, 68 (34%) had viral meningo-encephalitis, 15 (8%) had parasitic meningitis, 13 (7%) had cryptococcal meningitis, and other diagnoses were recorded in

32 (16%). AFB were neither seen nor cultured from any of the specimens from these patients. 132 adults had clinical TBM: 107 (81%) had bacteriological confirmation of the diagnosis, 15 (11%) had probable TBM, and 10 (8%) had possible TBM. 14/132 were infected with HIV.

4.3.2 Performance of ZN stain and culture

The sensitivity, specificity, positive and negative predictive values of CSF stain and culture before the start of ATC are presented in **Table 4.1.** AFB were seen in the CSF of 73/132 (55%) patients (73/143 specimens) before the start of ATC.

Table 4.1 Sensitivity, specificity, and positive and negative predictive values of CSF stain and culture before the start of ATC

	GOLD STANDARD		
	M.tb CSF culture	***Clinical diagnosis***	
	CSF ZN stain	**CSF ZN stain**	***M.tb* CSF culture**
Sensitivity	65/90 (72%)	73/143 (51%)	90/143 (63%)
Specificity	286/294 (97%)	241/241 (100%)	241/241 (100%)
Positive predictive value	65/73 (89%)	73/73 (100%)	93/93 (100%)
Negative predictive value	286/311 (92%)	241/311 (77%)	241/291 (82%)

After 2 days of treatment bacilli were seen in 9/50 (18%) patients (50 specimens), and in 5/73 (7%) patients (73 specimens) taken after 7 days of treatment. No bacilli were seen in the CSF after this time. AFB were seen in the CSF of 4 patients after the start of treatment in which the pre-treatment slides were negative. Therefore, bacteriological confirmation of the diagnosis of TBM was made by CSF stain in 77/132 (58%) during the first week of ATC.

M.tb was isolated from the CSF of 90/132 (68%) patients (90/141 specimens) before the start of treatment. ATC quickly reduced the likelihood of isolating an organism from the CSF: after 2 days *M.tb* was cultured from 18/50 (36%) adults (50 specimens), and 11/73 (15%) (73 specimens) after 7 days. Two adults had *M.tb* isolated after 30 and 60 days of treatment respectively: both of these organisms were resistant to isoniazid and streptomycin. CSF cultures were sterile from 13 patients in which bacilli were seen. Nine were in specimens taken before the onset of treatment, and 4/13 taken after the start of treatment. All these specimens came from patients with clinical features of probable TBM who responded appropriately to ATC, and were not considered false positive.

4.3.3 Variables associated with positive ZN stain and culture

The variables associated by univariate analysis with finding bacilli and i solating *M.tb* from the CSF analysis are presented in **Table 4.2**. These data show infection with HIV and increased CSF neutrophil percentage is significantly associated ($p<0.05$) with a positive stain and a positive culture. In addition, low CSF lymphocyte percentage, high

Table 4.2 Variables associated with positive CSF stain and culture before the start of ATC by univariate analysis (median and range).

	Bacilli seen	No bacilli	p-value	*M.tb* cultured	Sterile CSF	p-value
Age(years)	30(15-68)	34(15-79)	**0.302**	30(15-69)	36(15-79)	**0.129**
Male Sex	42/73(57%)	36/70(51%)	**0.464**	51/93(55%)	27/50(54%)	**0.923**
Grade 1	19/73(26%)	24/70(34%)		28/93(30%)	15/50(30%)	
Grade 2	36/73(49%)	32/70(46%)	**0.504**	45/93(48%)	23/50(46%)	**0.937**
Grade 3	18/73(25%)	14/70(21%)		20/93(22%)	12/50(24%)	
Hiv +	11/14(79%)	3/14(21%)	**0.030**	13/14(93%)	1/14(7%)	**0.022**
Hiv -	62/129(48%)	67/129(52%)		80/129(62%)	49/129(38%)	
Symptoms (days)	15(6-60)	17(5-60)	**0.302**	15(5-60)	15(5-51)	**0.274**
CSF Pressure (cm H_20)	23(5-41)	22(7-40)	**0.805**	23(5-40)	21(7-41)	**0.481**
CSF WCC	317(1-1880)	360(5-1750)	**0.678**	312(1-1880)	378(5-1750)	**0.453**
% neut	37(0-95)	27(0-84)	**0.048**	37(0-95)	20(0-81)	**0.004**
% lymphs	63(5-100)	71(16-100)	**0.083**	63(5-100)	80(19-100)	**0.009**
CSF Protein (mg/dl)	200(45-1800)	180(64-800)	**0.270**	200(45-1800)	161(64-700)	**0.069**
Lactate (mmol/l)	6.8(2.1-15.1)	5.9(1.1-16.4)	**0.181**	6.8(1.5-16.4)	5.5(1.1-9.8)	**<0.001**
Chloride (mmol/l)	104(81-129)	107(76-129)	**0.270**	101(76-129)	107(90-129)	**0.021**
CSF:blood glucose ratio	0.23(0.04-0.67)	0.29(0.04-0.67)	**0.157**	0.25(0.05-0.67)	0.25(0.04-0.55)	**0.923**
Volume(mls)	4.0(0.5-10)	3.0(0.2-12)	**0.096**	4.0(0.2-12.0)	3.0(0.4-8.0)	**0.236**

CSF lactate concentration, and low CSF chloride concentration, are significantly associated with a positive CSF culture.

When the same variables were compared by multivariate analysis using forward logistic regression, only the CSF:blood glucose ratio (OR 0.03, 95% CI 0.002-0.621, p=0.023) is independently predictive of seeing AFB in the CSF. The factors independently associated with the bacteriological confirmation of TBM (*M.tb* cultured from the CSF and/or AFB seen in the CSF) are presented in **Table 4.3**.

Table 4.3 Factors associated with the bacteriological confirmation of TBM before the start of ATC by forward logistic regression

Factor	Odds ratio	95% confidence interval	p-value
Duration of symptoms (days)	1.05	1.00-1.10	0.050
Volume of CSF (mls)	1.36	1.06-1.75	0.017
CSF neutrophils (% of WCC)	1.03	1.01-1.05	0.008
CSF lactate (mmol/l)	1.42	1.16-1.73	0.001
CSF:blood glucose ratio	0.03	0.02-0.62	0.023

The median time to see bacilli in the CSF before the start of treatment was 10 minutes (range 1-50). **Figure 4.1** presents a histogram of the times taken to see bacilli, and the cumulative percentages of positive slides as the time to examine the slide increases.

Figure 4.1 Time to see bacilli in the CSF before the start of treatment

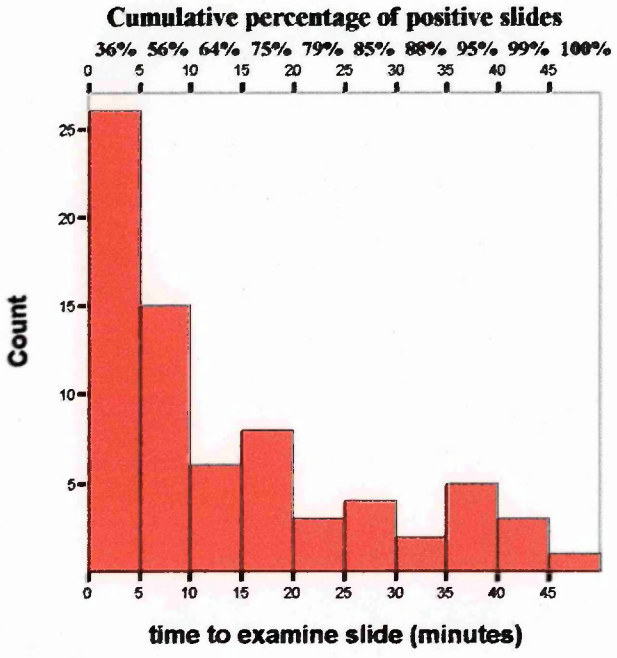
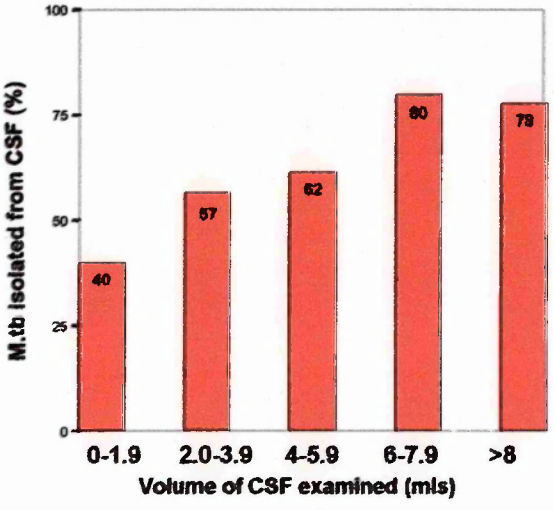


Figure 4.2 Volume of CSF submitted for culture, and the likelihood of culturing *M.tb*



The median volume of CSF examined was 4.0mls (range 0.2-12.0mls), and the median time to examine each slide was 20 minutes (range 1-50 minutes). The median volume of CSF submitted after starting ATC was 3.0mls (range 1.0-12.0mls). **Figure 4.2** shows the affect of increasing volume upon culture rates in HIV negative individuals before the start of treatment. *M.tb* was isolated from smaller CSF volumes from HIV-infected individuals (medians 1.5mls vs 4.0mls, p=0.001).

4.4 Discussion

The search for AFB in the CSF is widely believed to be too insensitive to assist in the management of many patients. The decision to start ATC cannot wait until *M.tb* has been cultured from the CSF; therefore many patients are treated for TBM on clinical suspicion alone. **Chapter 3** explores the possibility of improving clinical diagnostic methods, particularly in settings with limited diagnostic microbiology. However, this study shows that the performance of CSF stain and culture can be substantially improved and identifies both procedural and clinical variables that are associated with improved performance.

The isolation of *M.tb* from the CSF is the gold standard diagnostic test for TBM, but limited sensitivity has resulted in many authors replacing this gold standard with a set of clinical diagnostic criteria. This introduces problems when assessing the performance of old or new diagnostic tests: there are no universally accepted diagnostic criteria, and none have been prospectively assessed against the true gold standard of culture. This study

presents performance data using culture and clinical diagnostic gold standards (**Table 4.1**). Using the culture of *M.tb* from the CSF as gold standard, the sensitivity of CSF ZN stain before the start of ATC was 72%. This figure is similar to one reported by Kennedy (Kennedy DH *et al.*, 1979), although less than that reported by Stewart (Stewart SM, 1953). The excellent results reported by Stewart may be partly due to available ATC at the time of their study (pre-1953). As this study demonstrates, 4-drug ATC containing these two drugs rapidly sterilises the CSF: after 2 days the sensitivity of ZN stain and culture fell respectively to 18% and 36%, and to 7% and 15% after 7 days.

M.tb was only cultured from the CSF of 2 patients after 7 days of treatment, and both isolates were resistant to isoniazid and streptomycin. Rifampicin and isoniazid were unavailable during Stewart's study, which was performed in the 1940's, and early 1950's, and there was limited availability of streptomycin and PAS. Therefore, Stewart's ability to find AFB in the CSF in more than 90% may be due to repeated CSF sampling from patients on weakly active ATC (PAS and streptomycin alone) or no treatment at all. **Chapter 5** examines the impact of drug resistance on the isolation of *M.tb* from the CSF after the start of ATC.

The duration of treatment for TBM, and the potential toxicity of the drugs, mandates high diagnostic specificity. This study suggests both ZN stain and culture for *M.tb* in the CSF is highly specific. However, AFB were reported but not cultured in 8 specimens taken before the start of ATC. These may represent false positives: debris from poorly filtered carbol fuscin can mimic AFB, as can small scratches on the surface of the slide.

However, the stringent requirement of this study to see 2 or more bacilli before calling a slide positive makes this explanation unlikely. Also, the clinical features of all the patients were consistent with TBM and all responded appropriately to ATC. There may be two explanations for these sterile cultures. First, the concentration of bacilli in some specimens will be on the limit of detection for stain and culture (considered to be around 10^4 bacilli/ml (Collins CH *et al.*, 1997)): the chance distribution of bacilli between the divided deposit may result in a concentration insufficient for culture. The characteristic of *M.tb* in clinical specimens to stick together in clumps makes this explanation more tenable. Alternatively, the AFB seen in the CSF may not be viable.

Most patients with TBM present after many days of symptoms, and are often treated initially for suspected pyogenic infections. In particular, prior treatment in the community with fluroquinolone antibiotics is common in Vietnam. These drugs have bactericidal activity against *M.tb*, and could account for non-viable AFB in the CSF. The phenomenon of visible but non-cultivable AFB is well recognised in the sputum of partially treated pulmonary tuberculosis, and has also been reported in the CSF of patients with TBM (Bonington A *et al.*, 2000). In this study AFB were found but not cultured in the CSF from 4 patients after the start of treatment – all had *M.tb* isolated from the CSF before ATC. These organisms are presumed to represent dead bacilli.

Many authors have considered the low sensitivity of the bacteriological diagnosis of TBM to be due to low concentration of organisms in the CSF. As a consequence, the volume of CSF examined is believed to be critical to diagnostic performance; but there

are few data to support this assertion. This study shows that CSF volume is independently associated with bacteriological diagnosis (Odds ratio 1.36, 95% CI 1.06-1.75 p=0.017). **Figure 4.2** shows that *M.tb* was isolated from 80% of specimens when more than 6 mls of CSF were cultured, and from 40 % when less than 2 mls were cultured. Low concentrations of bacilli in the CSF often requires prolonged microscopy to confirm their presence, and this study showed that 20% of positive slides required more than 25 minutes microscopy to find AFB. However, the relationship between duration of microscopy and the number of positive slides was not linear (**Figure 4.1**): 36% slides were found to be positive after less than 5 minutes microscopy, but a further 45 minutes microscopy to confirm the remaining 64%. The reason for this relationship is likely to be due to microscopy technique and the characteristic of *M.tb* bacilli to stick together in clinical specimens. It was observed early in the study that AFB were most commonly found in the areas of the slide with the highest cellular density. Thereafter, these areas were identified first under low power and a careful search was made of them under high power. If this proved fruitless the rest of the slide was examined systematically. Directing the initial search for bacilli to areas most likely to contain them probably explains the high yield (56%) in the first 10 minutes of microscopy. However, these data also confirm the importance of prolonged microscopy (greater than 20 minutes) to the diagnosis in 25% of cases.

It may be difficult for laboratories with limited staff and resources to perform the level of microscopy required to achieve the best results. For this reason, clinical predictors of the

diagnosis of TBM, and the likelihood of achieving bacteriological confirmation, might be helpful in directing resources to specimens most likely to be positive. **Chapter 3** investigates whether TBM might be distinguished clinically from other causes of bacterial meningitis, and the algorithms described could be used to distinguish patients in whom careful microscopy and culture for *M.tb* is warranted. The variables described in **Table 4.3** could be applied to patients selected by these algorithms to further assess the likelihood of obtaining a bacteriological diagnosis: the search for AFB should be especially stringent when the patient has a long history, a CSF pleocytosis with a high percentage of neutrophils, a high concentration of CSF lactate, and a low CSF:blood glucose ratio.

The effect of HIV infection upon the performance of conventional bacteriology appears marked, although there were only 14 HIV-infected patients with TBM in this study. *M.tb* was isolated from significantly smaller CSF volumes from HIV infected individuals compared to those without the virus (medians 1.5mls vs 4.0mls, p=0.001). These data suggest these adults may have greater CSF bacterial concentrations, and the bacterial diagnosis may be easier to confirm. Other clinical variables independently associated with bacteriological diagnosis (**Table 4.3**) may also reflect bacillary concentration. If this is true these variables may also be associated with pathogenesis and outcome. These speculations are explored further in **Chapter 8**.

CHAPTER 5

THE MOLECULAR DIAGNOSIS OF TUBERCULOUS MENINGITIS

5.1 Introduction

Early reports suggested the amplification of nucleic acid specific to *M.tb* from the CSF of patients with TBM might improve upon conventional bacteriology (Kaneko K *et al.*, 1990; Shankar P *et al.*, 1991). A recent systematic review and meta-analysis of the accuracy of nucleic acid amplification (NAA) tests for the diagnosis of TBM concluded these tests were specific, but insensitive (sensitivity of commercial assays 56% [95% CI 46-66%]) (Pai M *et al.*, 2003). There are a number of reasons why these methods have failed to meet initial expectations. First, a wide variety of methods are reported, many are in-house, and conclusions regarding their general applicability are difficult. Second, few studies have been able to compare performance against a bacteriological gold standard, instead choosing a variety of clinical diagnostic criteria that have never been prospectively assessed. Lastly, TBM is rare in most settings capable of applying the required technology; consequently most published studies are small, reporting few patients with TBM, and lack the statistical power to demonstrate unequivocal improvements in performance.

The FDA of the United States have licensed two assays for the direct detection of *M.tb* nucleic acid in smear positive respiratory samples: the Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test (MTD) (Gen-Probe, San Diego, California) and the AMPLICOR Mycobacterium tuberculosis test (Roche Diagnostics, Indianapolis, Indiana). Neither of these kits is licensed for use on non-respiratory samples, and there are limited data regarding their performance using CSF. Studies using the Roche AMPLICOR assay suggest it does not improve upon microscopy for acid-fast bacilli in the CSF (Bonington A *et al.*, 1998; Brienze VM *et al.*, 2001).

The MTD is a target-amplified nucleic acid probe test for the *in-vitro* diagnostic detection of *M.tb* ribosomal ribonucleic acid (rRNA). The test uses Transcription-Mediated Amplification (TMA) and the Gen-Probe Hybridisation Protection Assay (HPA) to qualitatively detect rRNA from all organisms of the *M.tb* complex. It is a two-part test in which amplification and detection take place in a single tube. Initially, nucleic acids are released from mycobacterial cells by sonication. Heat is used to denature the nucleic acids and disrupt the secondary structure of rRNA. The TMA amplifies a specific rRNA target by transcription at a constant 42^0C, resulting in multiple copies of *M.tb* rRNA amplicon. *M.tb* specific sequences are detected by probing with chemiluminescent-labeled complimentary DNA. A luminometer is used to detect stable RNA:DNA hybrids.

Table 5.1 Published reports using Gen-Probe MTD test on CSF for the diagnosis of TBM

Report	Setting	MTD Kit[a]	CSF specimens	TBM CSF specimens	AFB in CSF	*M.tb from CSF*	MTD + ve	MTD sensitivity (95% CI)	MTD specificity (95% CI)
(Chedore P *et al.*, 2002)	Canadian reference lab	II	311	16	7	16	15	93% [b] (70-100%)	99% (98-100%)
(Baker CA *et al.*, 2002)	USA inner city hospital	II	29	9	0	5	5	56% (21-86%)	100% (86-100%)
(Lang AM *et al.*, 1998)	Dominican Republic [c]	I	84	19	0	5	4	33% (17-55%)	100% (94-100%)
(Gamboa F *et al.*, 1997a)	Spanish Hospital	I	22	8	0	8	5	63% (26-90%)	100% (77-100%)
(Gamboa F *et al.*, 1997b)	Spanish Hospital	I	17	8	0	8	5	63% (26-90%)	100% (66-100%)
(Ehlers S *et al.*, 1996)	German Hospital	I	51	6	1	Not given	4	67% (24-94%)	98% (87-100%)
(Pfyffer GE *et al.*, 1996)	Swiss Hospital	I	54	6	1	5	6	100% (54-100%)	96% (85-99%)
TOTALS			**568**	**72**	**9**	**47**	**44**	**61% (49-72%)**	**99% (98-100%)**

[a] I= original MTD kit , II= enhanced MTD. [b] no data on clinical diagnosis given [c] Assay done in USA on frozen

There have been seven published studies that report the use of MTD for the diagnosis of TBM (**Table 5.1**). These data suggest it may be more sensitive than conventional bacteriology and other NAA tests (pooled sensitivity of MTD 61%, 95% CI 49-72%), but the effect of anti-tuberculosis chemotherapy (ATC) upon the performance of conventional and molecular diagnostic methods is also uncertain. There is some evidence to suggest repeated sampling after the start of ATC improves the diagnostic yield of CSF ZN stain and culture (Kennedy DH *et al.*, 1979). There are also some data that suggest *M.tb* DNA can be detected in the CSF for at least 4 weeks after the start of treatment (Donald PR *et al.*, 1993), in which case molecular diagnostic techniques may be especially useful once treatment has started. Organisms resistant to first-line drugs may be more readily detected by all methods after treatment, but this issue remains unexamined in patients with TBM.

The aim of this study was test the hypothesis that NAA (by MTD) is more sensitive than conventional bacteriology for the diagnosis of TBM, and to investigate the impact of drug resistant *M.tb* upon the sensitivity of these methods after the start of treatment.

5.2 Methods

5.2.1 Clinical methods

As described in **Chapter 4**, a special laboratory diagnostic service for TBM was set up at the CRU, and the attending physicians were encouraged to submit 5-10mls of cerebrospinal fluid from any adult admitted to with possible TBM. The volume of each

CSF sample submitted to the service was recorded, and processed according to the methods described in **Chapter 2.** The supernatant was removed to leave a volume equivalent of 5 or 6 drops into which the deposit was vigorously re-suspended. The deposit was divided into three equal volumes for ZN staining, MTD (frozen at -70^0C), and culture.

The clinical and laboratory features, and the final diagnosis of each patient admitted to the CRU were recorded prospectively in individual study notes as described in **Chapter 2.** All patients with TBM were tested for antibodies to HIV.

Lumbar punctures were performed on days 2, 7, 30, 60, and 270 of treatment for TBM. The samples were treated by the same methods described above. CSF cell counts and biochemistry were performed by standard methods upon each sample, as were Gram's and Indian ink stains with culture for fungi and pyogenic bacteria.

5.2.2 Adaptation of the MTD for CSF

Pfyffer *et al.* have reported a low sensitivity when using the MTD on untreated spiked CSF, and demonstrated improved sensitivity following pre-treatment with SDS-NaOH (5 x 10^5 cells/ml detected if untreated vs. 2 x 10^2 cells/ml detected if treated) (Pfyffer GE *et al.*, 1996). These experiments were repeated prior to starting MTD testing. CSF from a patient with culture confirmed cryptococcal meningitis was divided into 450ul aliquots and spiked with 50ul 1/10 dilution series of a clinical *M.tb* isolate (in PBS) in triplicate. Each dilution series was then subjected to one of three protocols: centrifugation and

re-suspension in 450ul phosphate buffer according to the package insert protocol for sputum; centrifugation and re-suspension in 100ul phosphate buffer (in order to increase rRNA concentration in lysed sample); and pre-treatment according to the method of Pfyffer *et al.*

Briefly, dH_20 was added to the sample to make 500ul, followed by 500ul 3.16% SDS-1% NaOH. This was vortexed, incubated for 40 minutes, then neutralised with 500ul 1.43% H_3PO_4, and washed with 500ul dH_2O. The deposit was then re-suspended in 450ul phosphate buffer and subjected to MTD according to the manufacturers protocol. The effect of 1, 2, 3 and 4-hour amplification incubation times on sensitivity were also tested using spiked CSF. Positive and negative cell controls, and two additional phosphate buffer negative controls, were included with each run according to the manufacturers recommendations. Results were interpreted according to the manufacturers specifications: >500,000 RLU positive, < 30,000 RLU negative and 30,000- 499,999 deemed equivocal and re-tested.

5.2.3 Statistical analysis

The paired proportions of positive tests before and after treatment were compared by McNemar's test. Unpaired proportions were compared by the Chi Squared test. The analysis was performed using Stata 6.0.

5.3 Results

5.3.1 Adaptation of the MTD to CSF

The limit of detection for untreated spiked CSF was 3 x 10^3 cells/ml. Re-suspension of CSF in a lower volume of lysis buffer decreased the sensitivity to 3 x 10^4 cells/ml, and pre-treatment according to the method of Pfyffer *et al* improved detection to 30 cells/ml. Experimentation with the enzyme incubation period suggested 3-hours gave the best sensitivity. Consequently, the protocol for testing all the clinical specimens was adapted to include pre-treatment, and 3-hour enzyme incubation.

5.3.2 Specimens, clinical data and controls

A ZN stain, a culture for *M.tb,* and the MTD were performed on 341 CSF samples from 152 adults admitted to the ward. 262 specimens came from 73 adults treated for TBM, and serial samples taken before and after ATC were available from 59 patients.

CSF specimens from 14 adults who started ATC before the start of the study were also included. A bacteriological diagnosis was confirmed in 57/73 (78%), 12/73 (15%) had probable TBM, and 4/73 (7%) had possible TBM. The admission clinical and laboratory findings are shown in **Table 5.2**.

Table 5.2 Admission data from 59 adults with TBM included before the start of ATC

Variable	Number (%)/median (range)
Male sex	30 (50.8%)
Age (years)	33 (15-69)
HIV infection	5 (8.5%)
Disease severity: Grade I	17 (28.8%)
Grade II	29 (49.2%)
Grade III	13 (22.0%)
Duration of symptoms before ATC (days)	15 (5-35)
Volume of CSF (mls)	4.0 (1-12)
CSF opening pressure (cm H_2O)	23 (6-40)
CSF total white cells (x 10^6/ml)	461 (1-1750)
% neutrophils	27 (0-90)
% lymphocytes	73 (10-100)
CSF Protein (mg/dl)	169 (66-4700)
CSF Lactate (mmol/l)	5.8 (1.1-16.4)
CSF chloride	104 (84-129)
CSF:blood glucose	0.25 (0.05-0.67)
9-month survival	45 (76.3%)

The diagnosis of TBM was excluded in 79 adults (**Table 5.3**), and 79 admission CSF specimens from these patients served as negative controls.

Table 5.3 Diagnoses of control group

Diagnosis		**CSF samples**
Bacterial meningitis:		
i)	Confirmed [a]	13
ii)	Probable [b]	7
Pyogenic brain abscess		1
Viral meningo-encephalitis		22
Mumps meningitis		1
Eosinophilic meningitis		12
Cryptococcal meningitis		20
Subarachnoid hemorrhage		2
TOTAL		**79**

[a] either CSF gram stain or culture positive

[b] short history, cloudy CSF, mostly neutrophils, recovered without ATC

5.3.3 Diagnostic performance before ATC

The sensitivity of CSF ZN stain (52%) and MTD (38%) were not significantly different before the start of ATC (p=0.150). The sensitivity, specificity, and positive and negative predictive values for each of the tests are shown in **Table 5.4**. ZN stain was 52% sensitive (95% CI 40 to 64%) and 100% specific against a clinical diagnostic gold standard, and 57% sensitive (95% CI 41 to 72%), 90% specific against a culture gold standard. AFB were seen but not cultured from 8 specimens, all were from adults with evidence of extra-neural tuberculosis who responded appropriately to ATC. The MTD was 38% sensitive (95% CI 26 to 51%), 99% specific against a clinical gold standard, and 50% sensitive (95% CI 34 to 66%), 95% specific against culture gold standard.

Table 5.5 presents the numbers of discrepant and concordant positive results with ZN stain and MTD, and their combined sensitivity, before and after starting treatment. Before treatment, the MTD was negative in 20/34 (59%) ZN positive specimens, but the ZN stain was negative in 11/25 (44%) specimens positive by MTD. The combined pre-treatment sensitivity of ZN stain and MTD (ZN+ and/or MTD+) was 45/66 (68%), but only 14/45 (31%) specimens were positive by both tests.

There was one false positive MTD from the CSF of an adult believed to have viral meningo-encephalitis. They made a complete recovery in hospital without ATC, and the patient was contacted and found to be well 18-months after admission, excluding a diagnosis of TBM.

Table 5.4 Sensitivity, specificity, and positive and negative predictive values of CSF ZN stain, MTD, and culture *before* the start of ATC

	CLINICAL DIAGNOSTIC GOLD STANDARD			
	Sensitivity (%) [95% CI]	**Specificity (%) [95% CI]**	**Positive predictive value (%) [95% CI]**	**Negative predictive value (%) [95% CI]**
ZN	34/66 (52%) [39-64%]	79/79 (100%) [95-100%]	34/34 (100%) [90-100%]	79/111 (71%) [62-79%]
MTD	25/66 (38%) [26-51%]	78/79 (99%) [95-100%]	25/26 (96%) [80-100%]	79/120 (66%) [57-74%]
Culture	38/66 (58%) [45-70%]	79/79 (100%) [95-100%]	38/38 (100%) [91-100%]	79/107 (74%) [64-81%]
	CULTURE GOLD STANDARD[a]			
ZN	24/42 (57%) [41-72%]	71/79 (90%) [81-95%]	24/34 (71%) [52-85%]	79/97 (81%) [72-88%]
MTD	21/42 (50%) [34-66%]	75/79 (95%) [88-98%]	21/25 (84%) [64-95%]	79/97 (81%) [72-88%]
Culture	38/42 (90%) [77-97%]	79/79 (100%) [95-100%]	38/38 (100%) [91-100%]	79/83 (95%) [88-99%]

[a] ***M.tb* isolated from the CSF at any time before or after starting ATC**

Table 5.5 Number of positive tests before and after starting ATC with concordant and discordant results of ZN stain and MTD over the first 40 days of treatment

	Pre-ATC	**2-5 days**	**6-15 days**	**16-40 days**	**41-80 days**	**260-280 days**
Patients/specimens	*59/66*	*34/34*	*43/43*	*48/48*	*35/35*	*36/36*
ZN positive	34/66(52%)	8/34(24%)	2/43(5%)	0/48	0/35	0/36
MTD positive	25/66(38%)	14/34(41%)	12/43(28%)	2/48(4%)	0/35	0/36
95% CI of difference	-4 to 31%	-40 to 4%	-41 to –5%			
p value	0.150	0.146	0.013			
Culture positive	38/66(58%)	12/34(35%)	6/43(14%)	0/48	1/35(3%)	0/36
MTD + / ZN -	11/66(17%)	9/34(26%)	12/43(28%)	2/48(4%)		
MTD - / ZN +	20/66(30%)	3/34(9%)	2/43(5%)	0/48		
MTD+ / ZN +	14/66(21%)	5/34(15%)	0/43	0/48		
MTD+ and/or ZN +	45/66(68%)	17/34(50%)	14/43(33%)	2/48(4%)		

5.3.4 Diagnostic performance after starting ATC

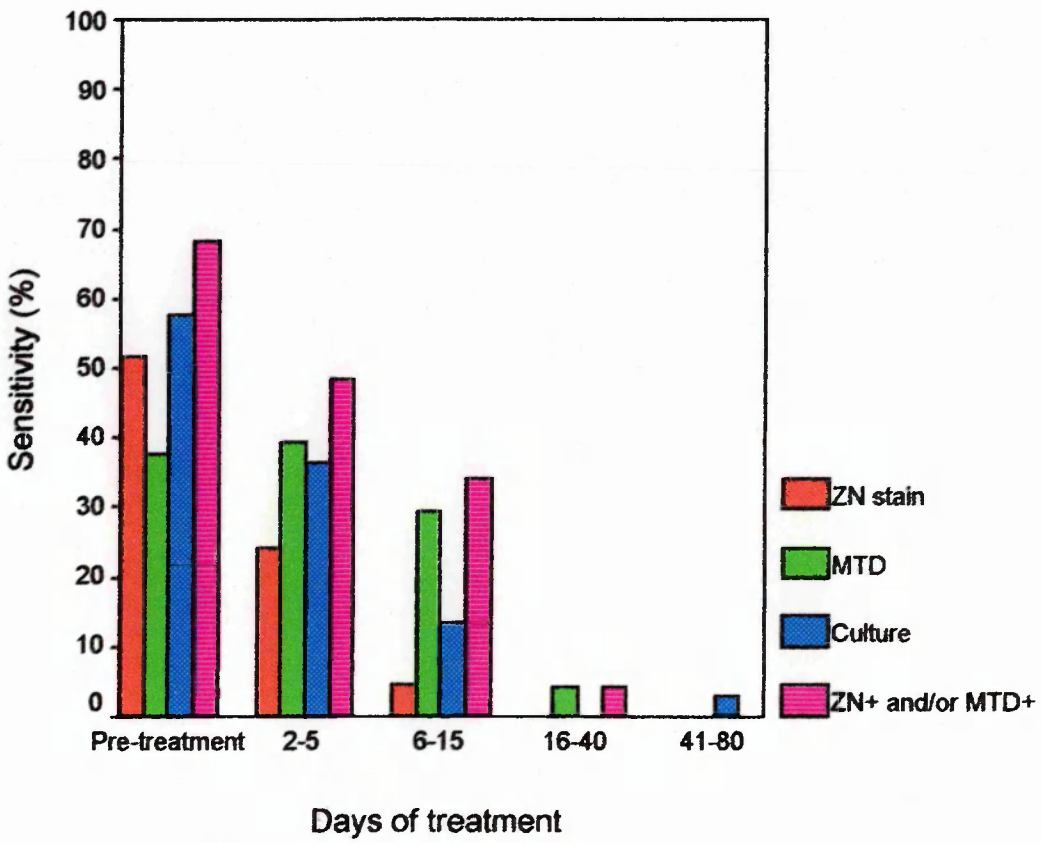
The sensitivity of ZN stains fell faster than MTD with treatment (**Table 5.5** and **Figure 5.1**). The sensitivity of MTD did not fall between days 2-5 of therapy, but was nearly 6 times more sensitive than ZN stain after 5-15 days of treatment (28% vs 5%, 95% CI of difference 5-41%, p=0.013). Agreement between ZN stains and MTD positive results worsened after the start of treatment: both tests were positive in 5/17 (29%) specimens positive by either test taken 2-5 days after treatment, and in 0/16 positive after 5 days. **Table 5.6** shows the number of new positive tests on repeated CSF samples from 59 patients before and after starting ATC.

Table 5.6 New positive tests before and after starting ATC on 59 adults with serial samples

	Pre-ATC	2-5 days	5-15 days	15-40 days	40-80 days	260-280 days	TOTALS (%) [95% CI]
ZN+	34	3	1	0	0	0	38/59 (64%) [51-76%]
MTD+	24	10	1	0	0	0	35/59 (59%) [46-72%]
ZN+ and/or MTD+	45	4	0	0	0	0	49/59 (83%) [71-92%]
Culture	38	3	1	0	0	0	42/59 (71%) [58-82%]

A repeat CSF day 2-5 improved the overall sensitivity of ZN stain by 3/33 (10%), and of MTD by 10/24 (42%). Repeated sampling resulted in a cumulative sensitivity of 38/59 (64%) for ZN stain, 35/59 (59%) for MTD, and 42/59 (71%) for culture against a clinical gold standard. The cumulative sensitivity of ZN stain and MTD combined was 49/59 (83%).

Figure 5.1 The sensitivity of ZN stain, MTD, culture, and combined ZN/MTD on 262 specimens taken before and after treatment from 73 patients with a clinical diagnosis of TBM



5.3.5 Impact of drug resistance on diagnostic performance

Table 5.7 presents the effect of resistance upon the performance of ZN, MTD and culture before and after the start of treatment. 10 isolates were resistant to streptomycin alone, one was resistant to isoniazid alone, 6 were resistant to both isoniazid and streptomycin, and one patient had multi-drug resistant *M.tb* (resistant to rifampicin, isoniazid, and streptomycin) and died before further samples could be taken. The 9-month survival for those with sensitive *M.tb* (22/28, 79%) was not significantly different from those with a drug resistant isolate (14/18, 78%).

Figure 5.2 The effect of drug resistance on the percentage of culture positive CSF before and after treatment

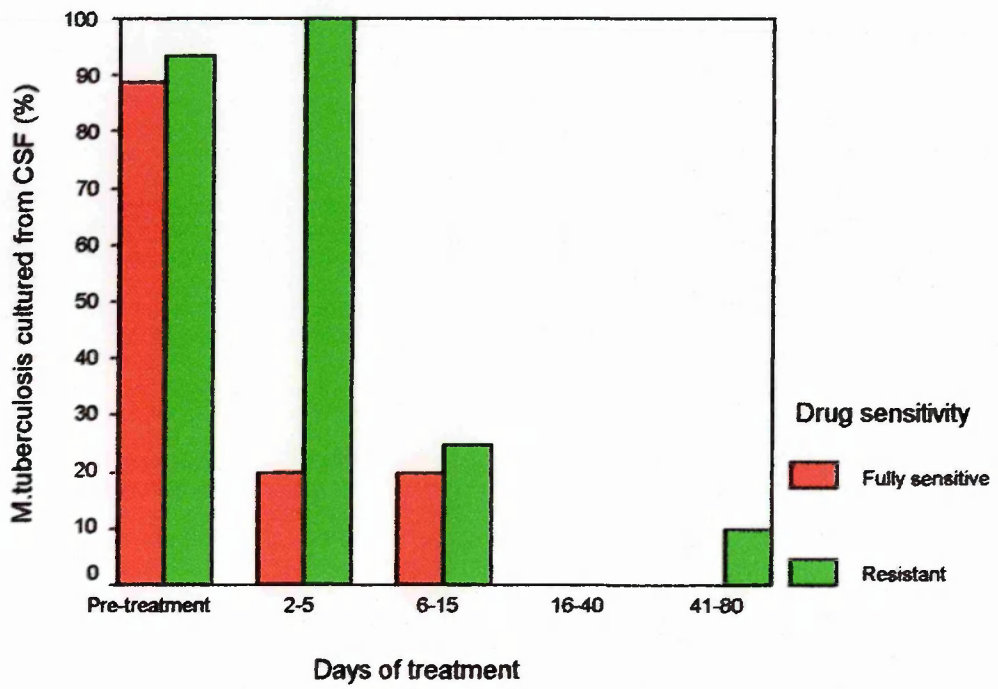


Table 5.7 The effect of drug resistance upon the performance of ZN, MTD, and culture before and after starting ATC

	Pre-ATC	2-5 days	5-15 days	15-40 days	40-80 days	260-280 days
			SENSITIVE M.tb[a]			
ZN positive	14/27(51.8%)	2/11(18.2%)	1/14(7.1%)	0/16	0/10	0/15
MTD positive	12/27(44.4%)	6/11(54.5%)	4/14(28.2%)	2/16(12.5%)	0/10	0/15
Culture positive	24/27(88.8%)	2/11(18.2%)	3/14(21.4%)	0/16	0/10	0/15
			DRUG RESISTANT M.tb[b]			
ZN positive	10/15(66.6%)	4/10(40%)	0/12	0/11	0/10	0/10
MTD positive	8/15(53.3%)	6/10(60%)	5/12(41.6%)	0/11	0/10	0/10
Culture positive	14/15(93.3%)	10/10(100%)	3/12(25%)	0/11	1/10(10%)	0/10

[a]Sensitive to all first-line ATC

[b] Resistant to 1 or more first-line ATC

Before the start of ATC there were no significant differences between the sensitivity of ZN stain, MTD, and culture in patients with sensitive or resistant *M.tb* (**Table 5.7**). After 2-5 days of treatment 2/11(18%) with sensitive *M.*tb were positive by ZN, compared with 4/10(40%) if resistant (p=0.269). Drug resistant *M.tb* was cultured from all (10/10) specimens after 2-5 days of ATC, compared with 18% (2/11) if fully sensitive (p<0.001) (**Figure 5.2**). The MTD was positive in 5/12 (42%) specimens 5-15 days after treatment if *M.tb* was drug resistant, and 4/14 (28%) if the isolate was sensitive (p=0.484).

5.4 Discussion

The detection of *M.tb* nucleic acid in the CSF has been possible for more than 10 years, but its role in the rapid diagnosis of TBM remains poorly defined. The aim of this study was to compare conventional bacteriology with the detection of *M.tb* rRNA in the CSF using the Gen-Probe MTD. The strength of the study is the large number of serial CSF samples taken before and after treatment from patients with a confirmed bacteriological diagnosis of TBM. The MTD was chosen because it is a universally available commercial test, and is recognised by the FDA to perform with sufficient sensitivity and specificity to be granted a license for use on smear negative sputum. There are no commercial tests currently with an FDA license for use on extra-pulmonary samples.

These data shows that before the start of treatment the sensitivity of ZN stain is greater than MTD (52% vs 38%) against a clinical diagnostic gold standard, although the difference is not significant (p=0.150) (**Table 5.4**).

It has long been recognised that the sensitivity of CSF ZN stain can exceed 50%, although modern laboratories and many textbooks rarely report this level of performance. **Chapter 4** shows that the sensitivity of conventional bacteriology is dependent upon the volume of CSF examined and the duration of microscopy. The factors that govern the performance of MTD are uncertain. The combined pre-treatment sensitivity of ZN stain and MTD was 45/66 (68%), with only 14/45 (31%) specimens positive by both tests (**Table 5.5**). Analysis of the discordant results between the tests reveal MTD was negative in 20/34 (59%) of ZN positive specimens, and the ZN stain was negative in 11/25 (44%) of MTD positive specimens (**Table 5.5**). It is difficult to understand why rRNA cannot be detected in CSF in which acid-fast bacilli have been seen. Pfyffer *et al* found that high concentrations of organisms were required in spiked CSF ($>5x10^5$cells/ml) to obtain a positive MTD, and suggested interference from unknown compounds in the CSF caused assay inhibition. These experiments have been repeated with similar results. Pfyffer also found that increasing the volume of sample used, pre-treatment with a denaturing agent such as SDS-NaOH, and increasing the amplification time from 2 to 3 hours could improve MTD sensitivity on CSF. Experimentation with the assay procedures concur with these findings, and suggests that inadequate rRNA extraction from small numbers of organisms in the CSF may be a factor in producing false negative MTD tests. The existence and impact of factors inhibitory to nucleic acid amplification in CSF remains unclear, but if present these factors are likely to be

substantially reduced by the detergent and washing actions of the pre-treatment protocol, and may partly explain the improvement in sensitivity produced by these steps. Freezing the CSF for later testing may also reduce sensitivity, although the high numbers of ZN stain negative, MTD positive specimens [11/25 (44%)] before treatment suggests an alternative explanation. There is a tendency for *M.tb* bacilli to stick together in clinical samples. This characteristic suggests bacilli are not evenly distributed through the sample and increases the chance of the divided deposit (for staining, MTD, and culture) containing different concentrations of bacilli. These sampling effects become particularly important when the CSF contains few bacilli and both tests are performing close to their limits of detection. This may explain the greater combined sensitivity of ZN stain and MTD: the larger the volume of sample tested by either test, the larger the chance of detecting *M.tb*.

Following the start of anti-tuberculosis treatment the sensitivity of ZN stain and culture fell rapidly (**Figure 5.1**). The sustained sensitivity of MTD results presumably from dead, non-cultivable bacilli. The value of repeated lumbar punctures after the start of treatment is still debated, but these data suggest they increase diagnostic sensitivity. The cumulative sensitivities of ZN stain, MTD, and the two tests combined are 64%, 59%, and 83% respectively, if they are repeated at least twice during the first 15 days of therapy (**Table 5.6**). The value of a repeat MTD during the first 5 days of treatment was particularly evident, as a repeat test detected 10/35 (29%) more cases than a single sample before

ATC. This should discourage dangerous delays in starting ATC, and may be useful to specialist centres admitting patients already started on ATC for unconfirmed TBM.

The impact of drug resistance upon bacterial clearance from the CSF is unknown, and this study could only assess the effect of streptomycin and isoniazid resistance. Evidence from pulmonary tuberculosis suggests these two drugs, and in particular isoniazid, are responsible for the majority of bactericidal activity in the first few days of treatment (Mitchison DA, 2000). This evidence, together with known excellent CSF penetration of isoniazid, suggests resistance may have a detrimental effect upon outcome in TBM, but this remains unsubstantiated. This study suggests that resistance to isoniazid and/or streptomycin does affect CSF bacterial clearance: *M.tb* was cultured from all specimens after 2-5 days of ATC if the organism was resistant, but from only 18% if the organism was sensitive ($p<0.001$) (**Figure 5.2**). The only organism to be cultured after 40 days of treatment was resistant to both drugs. The performance of ZN stain and MTD were not significantly different in patients with sensitive or resistant *M.tb*. Although these data suggest isoniazid and/or streptomycin resistance reduces early bactericidal activity in the CSF, a larger study is required to define whether a positive ZN and/or MTD after the start of ATC can predict resistance, and whether resistance to these agents worsens outcome. More useful still would be to predict multi-drug resistance by these methods, which carries a far worse prognosis and necessitates early intervention with second-line anti-tuberculosis drugs.

The time to get a result, and cost should also be considered when comparing CSF ZN stain with the MTD. These are especially important to busy laboratories in poorly resourced settings – where the majority of TBM occurs. A CSF ZN stain takes approximately one hour from lumbar puncture to result (15 minutes centrifugation, 15 minutes preparing the slide, and 30 minutes microscopy), although more than 50% of positive slides are confirmed within the first 10 minutes of microscopy (**Chapter 4**), and can be performed without addition to basic laboratory equipment. It takes a minimum of 6 hours to perform the CSF-modified MTD, regardless of result, and the laboratory must have a luminometer ($12,500 – Gen Probe, 2003), a sonicator ($495 – Gen-Probe, 2003), and must purchase test kits that cost over $20 per test (50 tests/kit; $1,100/kit: Gen-Probe, 2003).

In conclusion, this study shows that before the start of ATC a careful search for AFB in the CSF is as good or better than the MTD detection of *M.tb* rRNA for the diagnosis of TBM. The ZN stain is also faster and much less expensive. However, adding MTD to a careful microscopic examination of the CSF improves performance further, and detects nearly 70% of cases before the start of treatment. Once ATC has been started the MTD retains sensitivity longer than ZN stain or culture, and may be more useful in settings where pre-admission treatment with ATC is common. Resistance to one or more first-line anti-tuberculosis drugs slows bacterial clearance from the CSF, and significantly increases the likelihood of isolating *M.tb* from the CSF after 2-5 days of treatment. Repeated sampling after the start of treatment improves the sensitivity of all methods,

particularly MTD. These results suggest that MTD alone does not offer advantage over careful bacteriology before the start of treatment. However, performing a combination of these tests on repeated samples can improve sensitivity to greater than 80%, whilst retaining high specificity. Nevertheless, the fatal consequences of delayed treatment demand a more sensitive single diagnostic test.

Until such time as cheaper, more sensitive assays are developed, the rapid diagnosis of TBM in most laboratories should depend upon the meticulous and repeated search for acid-fast bacilli from a large volume (>6mls) of CSF. The clinical algorithms described in **Chapter 3** may identify patients most likely to have TBM, on whom the most time should be spent.

CHAPTER 6

THE PATHOPHYSIOLOGY OF TUBERCULOUS MENINGITIS

6.1 Introduction

The following two chapters are concerned with the pathogenesis of TBM and the factors that affect disease progression and outcome. TBM is the most dangerous form of infection with *M.tb*, but the pathogenesis remains unclear. An excessive intra-cerebral inflammatory response is considered responsible for the neurological damage, and adjunctive immunosuppression with corticosteroids has long been suggested, although the clinical benefit is uncertain (Prasad K *et al.*, 2000).

Studies in rabbits have suggested a central role for tumour necrosis factor-α (TNF-α) in the pathogenesis and progression of TBM (Tsenova L *et al.*, 1999), and thalidomide, an inhibitor TNF-α production, reduced mortality in these models (Tsenova L *et al.*, 1998). The mechanisms that underlie human disease are less well studied. There are differences between the cytokines expressed in the CSF of patients with viral, bacterial, fungal and tuberculous meningitides (Akalin H *et al.*, 1994; Glimaker M *et al.*, 1993). High concentrations of CSF TNF-α have been observed repeatedly in untreated bacterial meningitis (Akalin H *et al.*, 1994; Glimaker M *et al.*, 1993; Rydberg J *et al.*, 1995).

The CSF concentrations reported in TBM are lower, but probably persist for longer (Donald PR *et al.*, 1995). The role of soluble TNF-α receptors is uncertain. Higher TNF-α: receptors ratios in TBM compared with bacterial meningitis may reflect lower concentrations of biologically active TNF-α, which may prolong the inflammatory process (Rydberg J *et al.*, 1995). TBM is also characterised by increased CSF expression of other pro inflammatory cytokines interferon-γ (IFN-γ), interleukin-1β, interleukin-8 (IL-8), and the anti inflammatory cytokine interleukin-10 (IL-10) (Mastroianni CM *et al.*, 1997).

There is evidence of significant BBB breakdown in patients with TBM (Brown HC *et al.*, 2000). The mechanisms are probably multi-factorial, although the matrix metalloproteinases (MMPs) have recently been implicated (Matsuura E *et al.*, 2000). These molecules, secreted by monocytes and macrophages, are zinc-containing proteases that degrade extra-cellular matrix (Goetzl EJ *et al.*, 1996). They may cause cerebral injury by disrupting the BBB, facilitating leukocyte migration, and cleaving myelin proteins. Elevated CSF matrix metalloproteinase-9 (MMP-9) concentrations have been associated with focal neurological deficit and fatal outcome in Vietnamese adults with TBM (Price NM *et al.*, 2001). The activity of the specific tissue inhibitors of MMPs (TIMPs) may be equally important, in particular the balance between MMP-9 and TIMP-1 (its specific inhibitor).

There are few studies describing the expression of these molecules over the duration of treatment for TBM, and their relationship with BBB dysfunction, clinical progression, and outcome after 9 months of anti-tuberculosis chemotherapy (ATC).

The purpose of this study was to describe the effect of treatment upon the constituents of the CSF - in particular the CSF expression of a range of pro and anti-inflammatory molecules, MMP-9 and TIMP-1, and the integrity of the BBB - and to test the hypothesis that the cellular and molecular intra-cerebral immune response predicts death or survival from TBM. The relationship between CSF leucocytes and *M.tb* was explored using electron microscopy.

6.2 Methods

The adults in this study were all admitted to the CRU as described in **Chapter 2**.

6.2.1 Laboratory investigations

Commercial capture ELISA kits were used to measure CSF and blood concentrations of IFN-γ, TNF-α, IL-8, and IL-10 (OPTEIA ELISA, Becton Dickinson, San Jose, California, USA), and the CSF concentrations of MMP-9, TIMP-1, and the soluble TNF-α receptors 1 and 2 (R and D systems, Abingdon, UK). Details of the methods are provided in **Chapter 2**. The lower limits of detection were 10 pg/ml of IFN-γ, TNF-α, TNF-α R1, TNF-α R2, and IL-8; 15 pg/ml of IL-10; 200 pg/ml of TIMP-1, and 350 pg/ml of MMP-9. Albumin and IgG concentrations were measured in blood and CSF and their respective indices calculated as described in **Chapter 2**.

6.2.2 Electron microscopy of the CSF

A centrifuged CSF deposit containing numerous AFB by ZN stain was selected, fixed in 4% glutaraldehyde in 0.1M phosphate buffer, and transported to Oxford University, UK, for electron microscopy. On arrival the samples were post-fixed in 2% osmium tetroxide in phosphate buffer, dehydrated in ethanol, then treated with propylene oxide prior to embedding in Spurr's epoxy resin. 1μm sections stained with Azure A were examined by light microscopy to identify areas of interest. Thin sections of suitable areas were cut and stain with uranyl acetate and lead citrate prior to examination in a Jeol 1200EX electron microscope.

6.2.2 Statistical methods

Normally distributed variables were compared by the Student's t-test; all other continuous variables were compared by the Mann-Whitney U test. Analysis of correlation between variables was performed by Spearman's test. Variables associated with death ($p<0.1$) by univariate analysis were incorporated into multivariate logistic regression. Forward stepwise variable selection procedure was used with p-to-enter <0.05 and p-to-remove >0.1 to identify independent predictors of death. The analysis was performed using 'Statistical Product and Service Solutions' (SPSS) software version 10.0 (Microsoft, USA).

6.3 Results

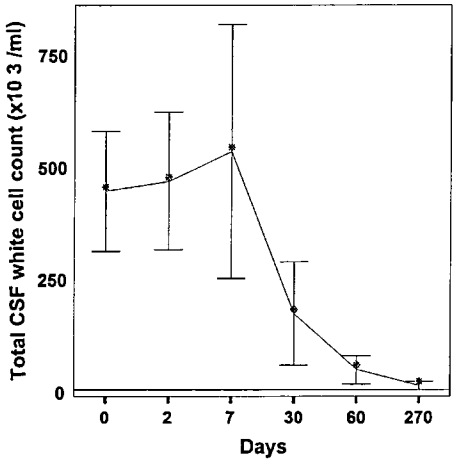
21 adults consented to enter the study between October 2000 and April 2001. At the start of treatment 8 were grade I, 6 grade II, and 7 grade III. None of the adults had antibodies to HIV. A diagnosis of TBM was confirmed (AFB seen in the CSF, or *M.tb* cultured from the CSF) in 15/21 (71%). One isolate was resistant to isoniazid, the rest were sensitive to all first-line agents. The CSF was sterile in 12/15 confirmed cases by day 3 of ATC, and in all by day 7. A diagnosis of TBM was highly probable in the unconfirmed cases: brain computerised tomography showed hydrocephalus and basal meningeal enhancement in 4/6, 3 of these had chest X-ray appearances consistent with active pulmonary tuberculosis. The remaining 2 adults were diagnosed with TBM on the basis of typical clinical and laboratory findings, and response to treatment. The median length of symptoms before admission was 18 days (range 6-45). 5/21 (24%) died before completing treatment: 2 after 2 days, the others after 13, 88 and 138 days of treatment. 2/16 (13%) survivors had severe neurological sequelae after 9 months of ATC. The rest made a complete recovery by the end of treatment. There was no relationship between length of symptoms before admission and disease severity or outcome.

6.3.1 Changes in CSF cellular and biochemical parameters over time

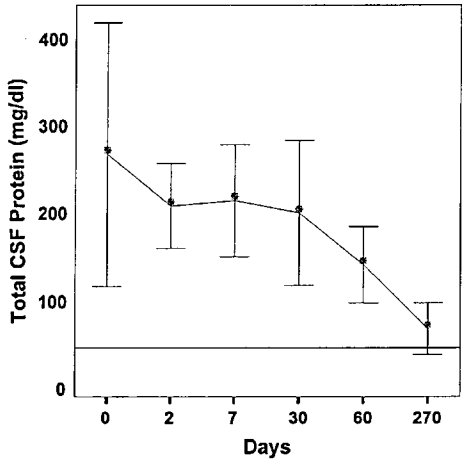
The effect of ATC upon CSF white cell counts (WCC), protein, glucose, and lactate concentration is shown in **Figure 6.1**. The mean WCC before treatment was 445 x 10^3 cells/ml (Standard Deviation [SD] 308); 533 x 10^3 cells/ml (SD 549) at day 7;

Figure 6.1 Mean values (with 95% confidence intervals) of CSF total WCC, protein, and CSF:blood glucose over treatment. *Continuous horizontal line represents the upper limit of the normal range.*

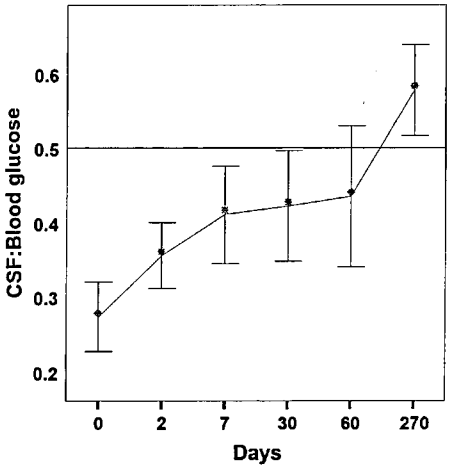
6.1 a Total CSF WCC



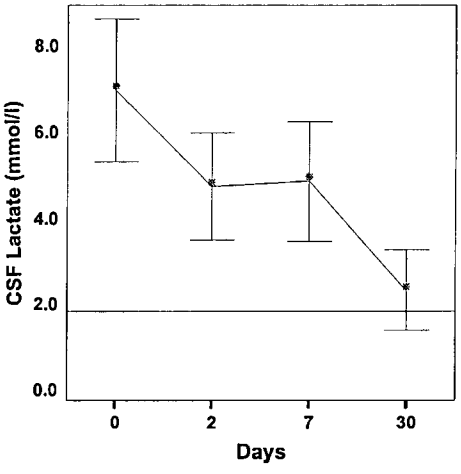
6.1 b Total CSF protein



6.1 c CSF:blood glucose



6.1 d CSF Lactate



173 x 10^3 cells/ml (SD 213) by day 30; and 48 x 10^3 cells/ml (SD 62) by day 60. CSF WCC was elevated (>5 x 10^3 cells/ml) in 8/13 samples taken after 270 days of ATC. CSF protein was elevated (>45 mg/dl) in many patients throughout treatment (**Figure 6.1b**). The mean concentration at the start of treatment was 266 mg/dl (SD 350) and was elevated in 5/13 (38%) patients at day 270.

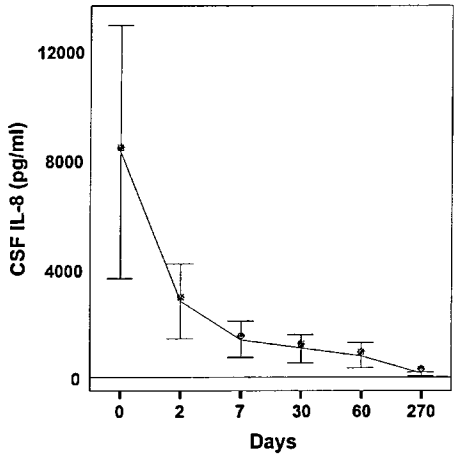
The ratio of CSF:blood glucose concentration was low (<0.5) in 23/24 measurements taken before the start of ATC (**Figure 6.1c**). After 270 days treatment the ratio was normal in 15/16 (94%) patients. The mean CSF lactate concentration was 6.9 mmol/l (SD 3.2) before treatment, 4.7 mmol/l (SD 1.6) on day 2, 4.8 mmol/l (SD 1.8) on day 7, and 2.3 mmol/l (SD 0.59) on day 30. CSF opening pressure was greater than 20 cm H_2O in 12/24 (50%) measurements taken from 21 patients before the start of ATC, 6/16 (38%) patients by day 7, 2/14 (14%) by day 30, 1/16 (6%) by day 60, and 0/13 by the end of treatment. Two patients had CSF pressures greater than 40 cm H_2O at the start of treatment both of whom died.

6.3.2 CSF and blood cytokines

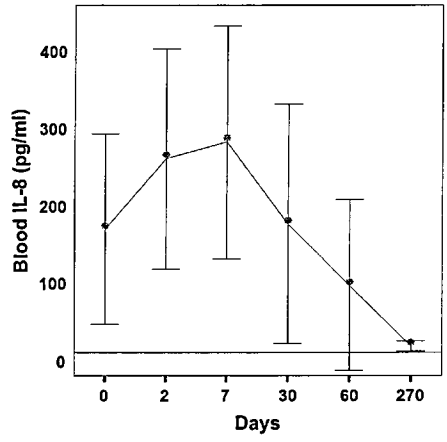
The CSF before treatment contained elevated concentrations of IL-8 (mean 8297 pg/ml, SD 9640), and IFN-γ (mean 708 pg/ml, SD 887) compared with values at the end of treatment (**Figure 6.2**). CSF concentrations of TNF-α were lower than those of IL-8 and IFN-γ (mean 66 pg/ml, SD 108) and undetectable in 15/17 (88%) adults at day 7 of treatment.

Figure 6.2 Mean concentrations (with 95% confidence intervals) of CSF and blood pro-inflammatory cytokines over treatment. *The horizontal line represents the limit of detection of the assay. The concentration of IFN-γ and TNF-α in sera was below the limit of detection for the assays, and is not included in the figure.*

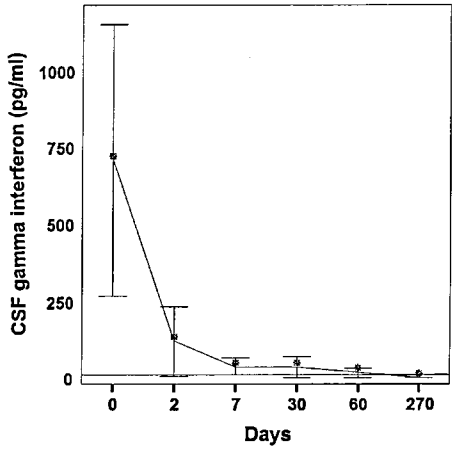
6.2 a CSF IL-8



6.2 b Blood IL-8



6.2 c CSF IFN-gamma



6.2 d CSF TNF-alpha

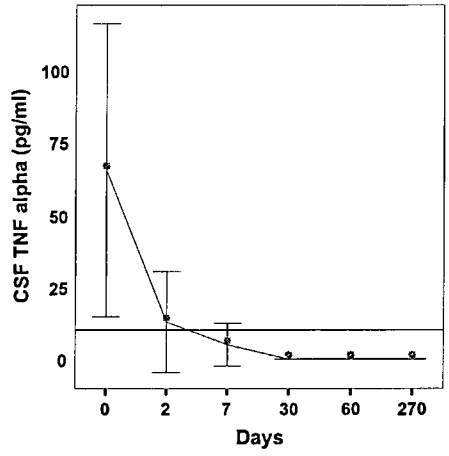
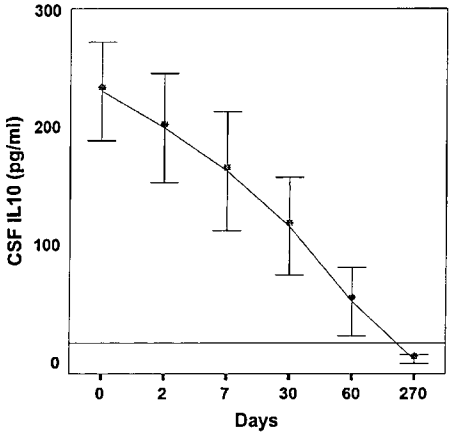
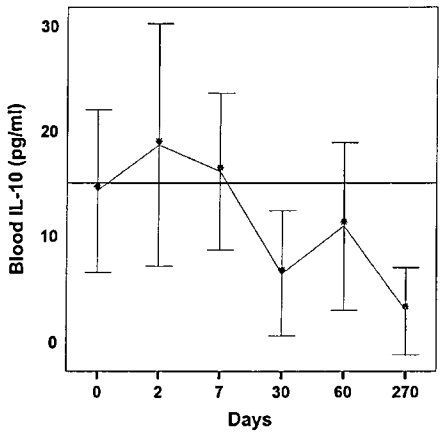


Figure 6.3 The mean concentrations (with 95% confidence intervals) of IL-10 and TNF-alpha receptors 1 and 2 over treatment. *The horizontal line represents the limit of detection of the assay.*

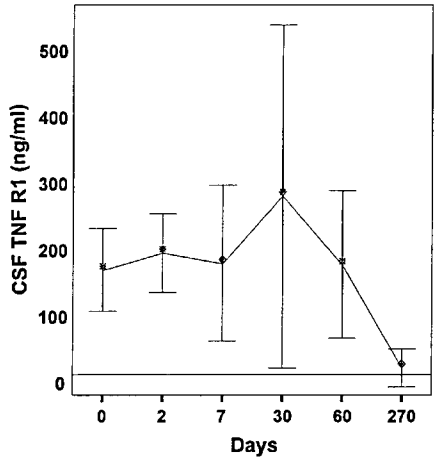
6.3a CSF IL-10



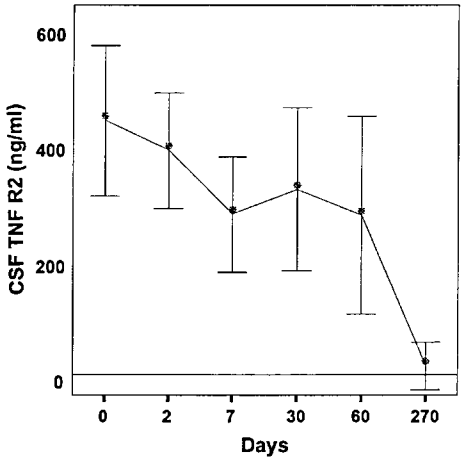
6.3b Blood IL-10



6.3c CSF TNF-alpha receptor 1



6.3d CSF TNF-alpha receptor 2



In contrast, concentrations of TNF-α receptors R1 and R2 were easily detectable after 60 days of treatment, falling to respective mean concentrations of 20 ng/ml and 25 ng/ml at the end of treatment (**Figure 6.3c and 6.3d**). Before treatment the mean concentration ratios of R1: TNF-α, and R2: TNF-α were 2.8 (0.24-11.5) and 7.7 (1.2-19.2) respectively (molar ratios 8.2 [SD 10.9] and 22.9 [SD 18.6]). These values rose to 9.9 (SD 12.6) and 18.1 (SD 20.1) by day 2, although only 3 patients had detectable concentrations of TNF-α at this time (molar ratios 29.2 [SD 37.1] and 53.6 [SD 59.5]). CSF concentrations of IL-10 fell with treatment to undetectable concentrations at 9 months (mean 2 pg/ml, range 0-14) (**Figure 6.3a**).

6.3.3 CSF matrix metalloproteinases

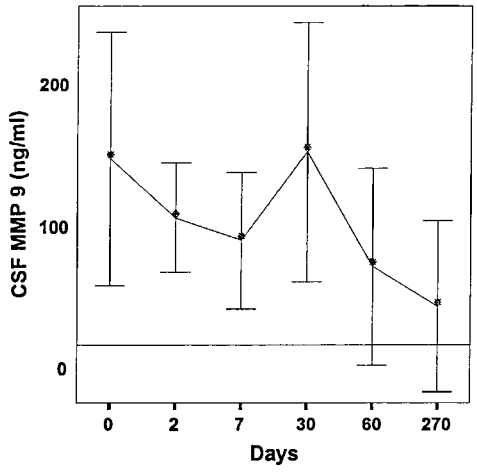
Figure 6.4 shows the mean CSF concentration of MMP-9 and TIMP-1 over treatment. Before treatment the mean CSF concentrations of MMP-9 and TIMP-1 were 146 ng/ml (SD 186) and 463 ng/ml (SD 192) respectively, which fell with treatment to 70 ng/ml (SD 139) and 269 ng/ml (SD 213) by day 60.

6.3.4 Albumin and IgG indices

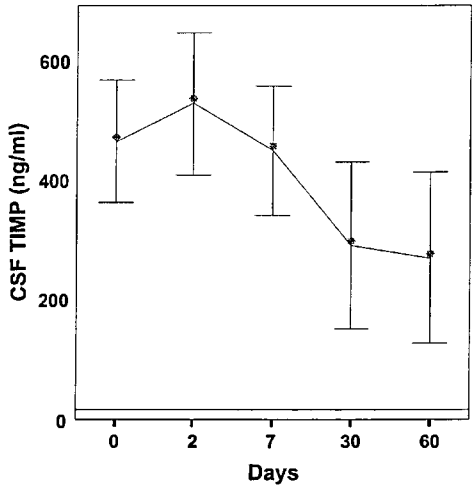
The albumin and IgG indices provide a useful measure of the extent of BBB permeability, and the serial values are presented in **Figure 6.4**. There is evidence of breakdown in the BBB throughout the first 60 days of treatment. Only at 9 months are the means of both indices within the normal range.

Figure 6.4 Mean CSF concentrations (with 95% confidence intervals) of MMP-9 and TIMP-1 (*the horizontal line represents the limit of detection of the assay*), and Log^{10} of the Albumin and IgG indices over treatment (*normal ranges indicated by horizontal lines*).

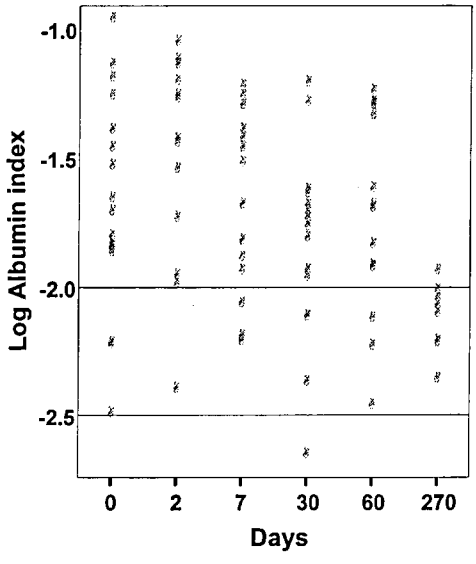
6.4a CSF MMP-9



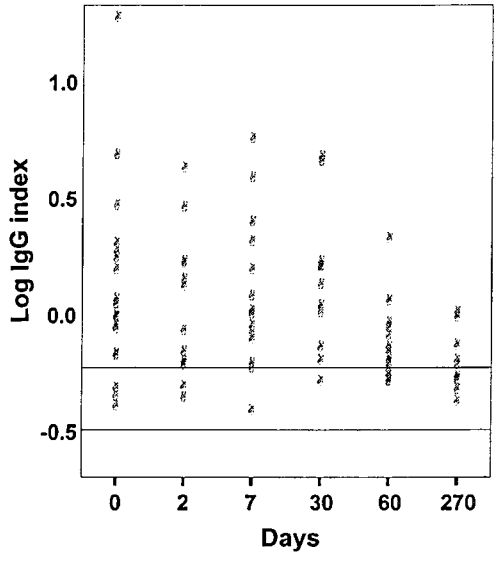
6.4b CSF TIMP-1



6.4c Log^{10} of the Albumin index



6.4d Log^{10} of the IgG index



6.3.5 Prognosis

Variables from those who died before the end of treatment were compared with those who survived (**Table 6.1**). Before treatment only CSF lactate concentration was significantly higher in those who died (p=0.029, 95% CI for the difference 0.4 to 6.8 mmol/l). When all values from day 0-7 were included the following were significantly associated with death: lower CSF WCC (95% CI –68 to –584, p=0.014), higher CSF % lymphocytes (95% CI 4 to 33, p=0.013), higher total CSF lymphocyte counts (95% CI 3 to 369, p=0.047), higher CSF lactate (95% CI 1.4 to 5.3, p=0.001), and lower CSF glucose:blood ratio (95% CI –0.04 to –0.19, p=0.003). Lower total CSF neutrophil counts were also associated with death (p=0.025).

Multivariate analysis was performed to predict variables independently associated with death. CSF lactate concentration was associated with death (Odds ratio 1.6, 95% CI 0.97-2.64, p=0.065) before treatment, and CSF WCC (Odds ratio 0.98, 95% CI 0.97-0.99, p=0.027) was associated with death over the first 7 days of treatment.

The same analysis was performed for those presenting with and without coma, and with or without focal neurological signs. None of the variables were significantly associated with these parameters (data not presented).

Table 6.1 CSF variables associated with death (p<0.10). Mean, standard deviation (in parentheses), and 95% CI of difference shown for normally distributed variables. The median, and range (in parentheses) is given for all others.

Variable	Survived N=16	Died N=5	95% CI of difference	P value
	CSF before treatment			
Total WCC (x10^3/ml)	509 (289)	213 (284)	-7 to + 599	0.055
% lymphocytes	66 (21)	87 (13)	-42 to + 1	0.058
% neutrophils	31 (23)	13 (13)	-4 to + 42	0.098
Lactate (mmol/l)	5.9 (2.6)	9.5 (3.3)	-6.8 to –0.4	0.029
CSF:blood glucose	0.30 (0.11)	0.20 (0.11)	-0.007 to +0.2	0.067
TIMP (ng/ml)	433 (187)	674 (23)	-532 to +51	0.098
Total neutrophils (x10^3/ml)[a]	108 (2-972)	3 (0-126)		0.062
	All CSF day 0-7			
Total WCC (x10^3/ml)	534 (388)	207 (262)	68 to 584	0.014
% neutrophils	29 (22)	15 (15)	-0.1 to 29	0.052
% lymphocytes	67 (22)	86 (15)	-33 to –4	0.013
Total lymphocytes (x10^3/ml)	336 (276)	150 (180)	3 to 369	0.047
Lactate (mmol/l)	5.0 (2.1)	8.4 (3.1)	-5.3 to –1.4	0.001
CSF: blood glucose	0.36(0.12)	0.24(0.09)	0.04 to 0.19	0.003
Total neutrophils (x10^3/ml)[a]	85 (0-1206)	6 (0-297)		0.025
MMP-9 (ng/ml)[a]	198 (0-395)	392(0-784)		0.058

[a] Not normally distributed: Mann-Whitney test used.

6.3.6 Correlation between variables

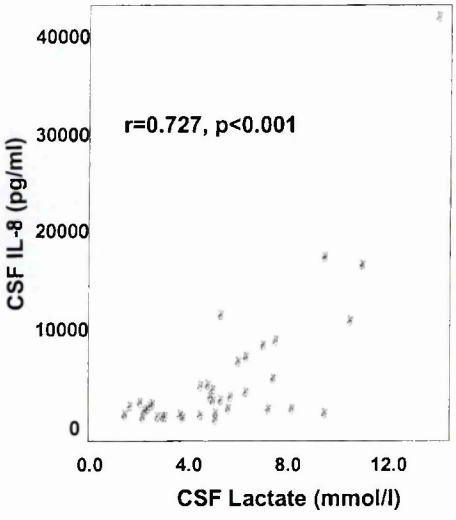
This analysis focused upon variables associated with death, only using data from the first 7 days of treatment. **Figure 6.5** presents the relationships between CSF lactate, CSF IL-8, and CSF IFN-γ. CSF lactate was correlated with CSF IL-8 ($r=0.727$, $p<0.001$), CSF TNF-α ($r=0.584$, $p<0.001$), CSF IFN-γ ($r=0.758$, $p<0.001$), and CSF TIMP-1 ($r=0.521$, $p=0.002$). CSF IL-10 concentration was correlated with CSF WCC ($r=0.497$, $p=0.001$), and IgGI ($r=0.527$, $p=0.001$). CSF:blood glucose ratio was weakly correlated with CSF IL-8 ($r=0.387$, $p=0.004$), CSF WCC ($r=0.284$, $p=0.031$), IgGI ($r=0.327$, $p=0.026$) and CSF lactate ($r=-0.351$, $p=0.039$). MMP-9 levels were correlated with CSF IL-8 ($r=0.476$, $p=0.001$), IFN-γ ($r=0.513$, $p=0.001$), with weak association between TIMP-1 ($r=0.320$, $p=0.041$), CSF protein ($r=0.298$, $p=0.034$) and CSF lactate ($r=0.362$, $p=0.042$). CSF concentrations of TIMP-1 were correlated with CSF lactate ($r=0.502$, $p=0.009$), CSF IL-8 ($r=0.5$, $p=0.001$), and IFN-γ ($r=0.574$, $p=0.001$). No significant relationship was found between MMP-9, TIMP-1 and the AI and IgGI, or between CSF neutrophil count, CSF IL-8 concentration, and the length of symptoms before treatment.

6.3.7 Electron micrographs of the CSF

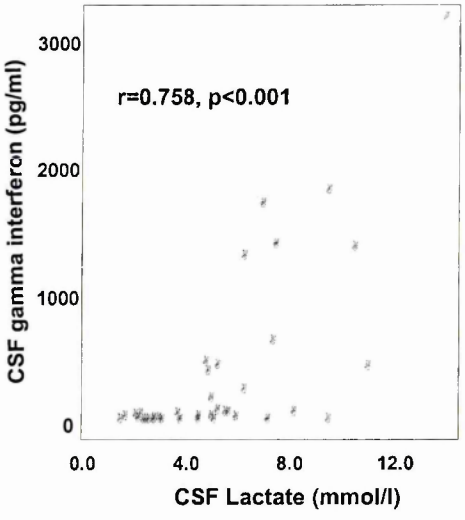
Figure 6.6 presents the electron micrographs taken, and demonstrates extra (**6.6 a**) and intra-cellular bacilli (**6.6 b, c, d**). Bacilli are seen within the phagasomes of neutrophils.

Figure 6.5 The relationship between CSF lactate, IL-8, and IFN-γ during the first 7 days of treatment, giving correlation coefficient (r) and p value by Spearman's test of correlation.

6.5 a The relationship between CSF lactate and CSF IL-8.



6.5 b The relationship between CSF lactate and IFN-γ.



6.5 c The relationship between CSF IL-8 and IFN-γ.

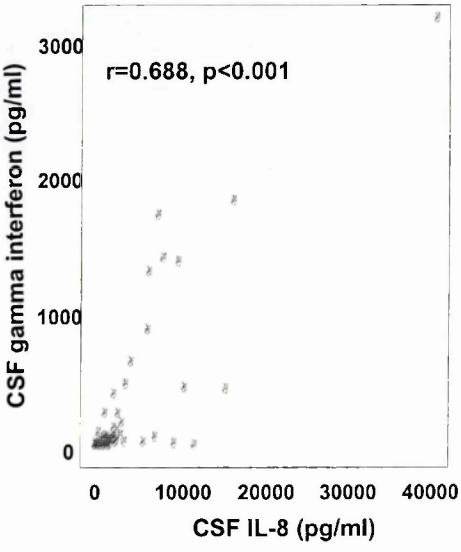
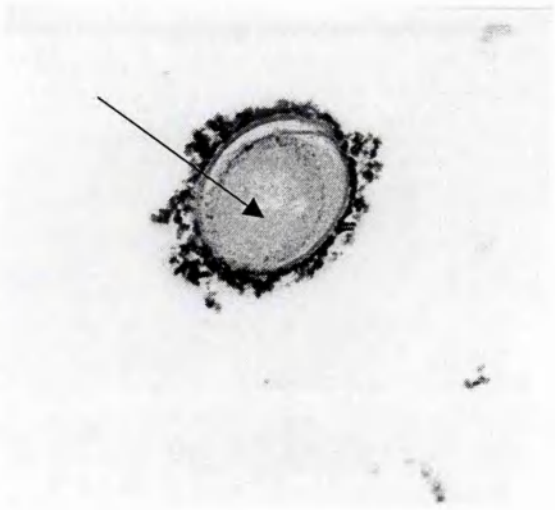
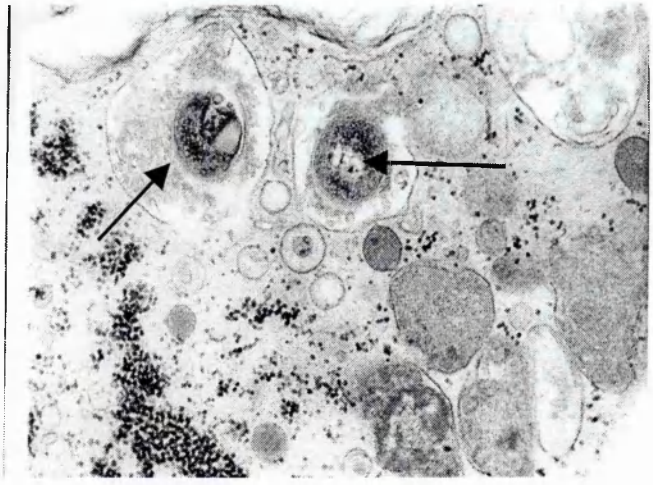


Figure 6.6 Electron micrographs of CSF deposit containing AFB from an adult with culture-confirmed TBM showing bacilli in the phagosomes of neutrophils

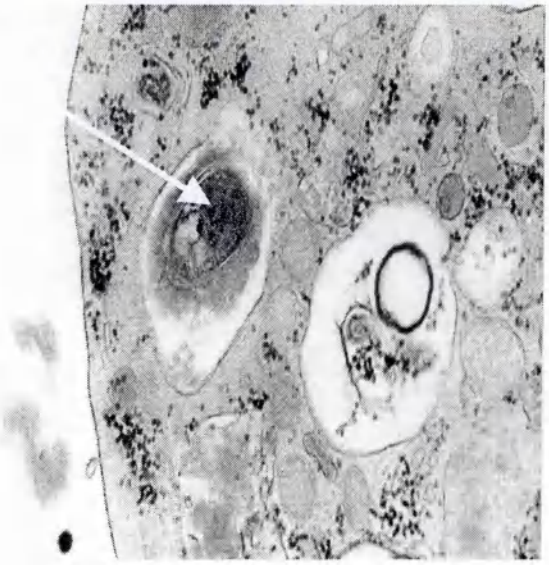
6.6 a Extra-cellular bacillus (arrow) (x 40,000)



6.6b Bacilli within the phagasomes of neutrophils (arrows) (x 30,000)



6.6c Bacillus within neutrophil (arrow) (x 30,000)



6.6d Bacillus within neutrophil (arrow) (x 12,000)

6.4 Discussion

This study followed 21 HIV negative adult patients throughout their treatment for TBM (none of the patients received corticosteroids). The concentrations of various pro and anti-inflammatory indices were recorded on serial CSF samples, the timing of which reflects the clinically important periods of the disease. The CSF specimen taken at the end of ATC (9 months) serves as an important indicator of successful treatment, and as a control for those samples taken earlier in the infection.

Ninety per cent of deaths from TBM occur in the first month of treatment (Girgis NI *et al.*, 1998). Deaths after this period are usually caused by complications arising from neurological sequelae, such as sepsis originating from the respiratory or urinary tract. Prognosis is dependent upon starting ATC before the onset of coma (Kennedy DH *et al.*, 1979). These facts suggest the inflammatory response immediately before and after starting ATC is critical to the outcome of the patient. Previous studies have shown CSF parameters respond slowly to treatment (Lepper MH *et al.*, 1963; Schoeman JF *et al.*, 2001), and the requirement for 6 to 12 months chemotherapy suggests a prolonged inflammatory response.

In this study blood and CSF samples taken before starting ATC reveal a highly compartmentalised immune response. Concentrations of CSF IL-8 were approximately 40 times that of blood, and neither IFN-γ nor TNF-α were detectable in blood despite easily detectable concentrations in the CSF (**Figure 6.2**). IL-8 is an inflammatory chemokine that is produced by many cell types and functions as a chemo-attractant for

neutrophils and a subset of T lymphocytes. When macrophages phagocytose *M.tb* in vitro, they express IL-8 in a TNF-α and IL-1β dependent manner (Zhang M *et al.*, 1995). IL-8 has been detected in plasma (Juffermans NP *et al.*, 1999) and bronchoalveolar lavage fluid (Kurashima K *et al.*, 1997) from patients with pulmonary tuberculosis and, as observed in this study, remained detectable for many months. The mechanisms driving prolonged expression of IL-8 in a disease not characterised by a strong neutrophilic response is unknown.

IFN-γ and TNF-α are recognised to be key cytokines in the control of *M.tb* infection (Flynn JL *et al.*, 2001). T cells and natural killer (NK) cells produce IFN-γ in response to *M.tb* infection, but absolute levels may be an unreliable correlate of protection (Zhang M *et al.*, 1995). This study found high concentrations of CSF IFN-γ before treatment but there was no association with outcome. Animal studies suggest TNF-α is central to TBM pathogenesis as TNF-α inhibition improves outcome (Tsenova L *et al.*, 1998). This study, as in previous reports, found low pre-treatment concentrations of CSF TNF-α, and there was no association between CSF TNF-α concentration and death. However, the biological activity of TNF-α may depend upon the relative concentrations of its soluble receptors, which may antagonise the biological effects of TNF-α at high concentrations, and promote them at low concentrations (Aderka *et al*, 1992). In this study the mean pre-treatment concentration ratios of soluble TNF-α receptors R1:TNF-α, and R2:TNF-α, were 2.8 and 7.7 respectively, compared to 27.2 and 28 reported by Rydberg et al (Rydberg J *et al.*, 1995). This suggests larger biologically active fractions

of TNF-α in our group of patients, although it is unclear whether the CSF in Rydberg's study was taken before or after treatment. This study suggests treatment reduces the CSF concentrations of TNF-α rapidly, whilst slowly reducing the receptor concentrations. By day 2 of treatment the ratios of receptors to TNF-α increased to 9.9 and 18.1. This may have the paradoxical effect of prolonging the biological activity of TNF-α despite falling CSF concentrations. Further investigation is required to address this hypothesis.

In bacterial meningitis it is hypothesised that bacterial lysis, induced by treatment with antibiotics, may contribute to the inflammation in the subarachnoid space and lead to a worse outcome (Tauber MG *et al.*, 1985). The same may be true for TBM, and has been a rationale behind adjuvant corticosteroids for many years. This study provides little support for this hypothesis. Only mean CSF WCC rose in the first 7 days of treatment (**Figure 6.1a**), and in all patients, regardless of outcome, ATC produced a rapid reduction in the CSF concentrations of IL-8, TNF-α, and IFN-γ (**Figure 6.2**). This response is in contrast to the kinetics of the CSF WCC, CSF protein, CSF:blood glucose ratio, CSF IL-10, CSF TNF-α receptors (**Figure 6.3**) and CSF MMP-9 and TIMP-1 (**Figure 6.4**), all of which were either detectable, or above the normal range, after 60 days of chemotherapy. Evidence for the longevity of the immune response is supported by the extent and duration of the BBB breakdown (**Figure 6.5**). These data suggests there may be two phases to the inflammatory response in TBM. The first is characterised by high CSF concentrations of IL-8 and IFN-γ, and lower concentrations of TNF-α, and is rapidly attenuated by ATC. The severity of this phase can be assessed by the concentration of

CSF lactate. The second phase is characterised by a persistent inflammatory response and breakdown in the BBB despite treatment, and may be maintained by the continued activity of CSF TNF-α soluble receptors, and the relative concentrations of MMP-9 and TIMP-1.

This study has limited power to associate the concentrations of any of these variables with death. However, CSF lactate concentration appears to be a good guide to prognosis: concentrations before and during the first 7 days of treatment were significantly higher in those who died (95%CI 1.4-5.3, p=0.001). Lactate is produced in all tissues in response to hypoxia. TBM causes an obliterative vasculitis with ischaemia, and often infarction (Dastur DK *et al.*, 1995), and CSF lactate concentration may reflect the severity of this process. CSF IL-8, IFN-γ, TNF-α, MMP-9, and TIMP-1 were all significantly correlated with CSF lactate suggesting these molecules might be associated with the pathogenesis. The CSF cellular response also appears significant to outcome: death was associated with a lower CSF WCC by univariate (95% CI –68 to –584, p=0.014) and multivariate analysis (Odds ratio 0.98, 95% CI 0.97-0.99, p=0.027). This is a surprising finding: previous reports have associated low CSF white cell counts with older age and HIV infection (Karstaedt AS *et al.*, 1998), suggesting an attenuated CSF cellular response as part of a systemic immune paresis. The phenotype of the cellular response may also carry prognostic significance. Survival was associated with a lower percentage of lymphocytes, and a higher percentage of neutrophils (**Table 6.1**). The electron micrographs of the CSF show bacilli phagocytosed by neutrophils (**Figure 6.6**) and although it is impossible to

infer functional activity from these pictures, together with associations between neutrophils in the CSF and survival and the likelihood of isolating bacilli, these findings suggest neutrophils may have a more important role in the early response to *M.tb* cerebral infection than previously thought. Murine studies have suggested neutrophils may have a protective role against *M.tb* infection (Pedrosa J *et al.*, 2000), but further study is needed to clarify their function.

The correlation between total CSF WCC and CSF IL-10 concentration may also be significant. It is tempting to speculate that IL-10 derived from infiltrating white cells might mediate immunosuppressive activity in the subarachnoid space. Before these hypotheses can be addressed, the functional phenotype of the CSF white cells needs to be characterised. There is evidence gamma delta T-cells may be important in the CSF response to *M.tb* (Dieli F *et al.*, 1999), but serial flow cytometric analysis of CSF is required to further define the cellular immune response. Larger studies are needed to further characterise the molecules and cells important to pathogenesis and outcome. These may suggest alternative therapeutic approaches that might supersede the blind use of corticosteroids.

CHAPTER 7

OTHER PROGNOSTIC FACTORS IN TUBERCULOUS MENINGITIS

7.1 Introduction

The mortality from TBM remains high, even in those with uncomplicated disease who are treated early. This may be partly explained by host immunity to *M.tb* and the intra-cerebral inflammatory response, as discussed in **Chapter 6.** However, survival may also depend upon the variable penetration of individual drugs to the site of disease and their differing ability to alter the disease pathogenesis. This chapter focuses upon other factors that may have a significant impact upon disease progression and outcome, in particular HIV infection, drug resistance, and bacterial genotype.

Isoniazid and rifampicin form the core of successful short course chemotherapy for pulmonary tuberculosis. Isoniazid has potent early bactericidal activity and kills about 95% of pulmonary organisms within 2 days of treatment. Rifampicin then assumes the major bactericidal role, and is believed responsible for the eradication of persisting organisms in the continuation phase of treatment (Mitchison DA, 2000). However, the addition of rifampicin to isoniazid in the treatment of TBM has not appeared to improve outcome or reduce the length of treatment required (Humphries MJ *et al.*, 1990; Ramachandran P *et al.*, 1986; Ramachandran P *et al.*, 1989).

The limited passage of rifampicin across the blood-brain barrier may partly explain this observation (Ellard GA *et al.*, 1993). In contrast, isoniazid penetrates freely into CSF (Ellard GA *et al.*, 1993) (see **Table 1.4**). This property, in combination with potent early bactericidal activity, suggests isoniazid is critical for successful TBM treatment. These features have prompted some to suggest higher doses (10-20mg/kg) of isoniazid may be indicated for the treatment of TBM to compensate for the uncertain penetration and efficacy of the other drugs in the regimen (Donald PR *et al.*, 1998; Humphries M, 1992).

Resistance to first-line anti-tuberculosis agents is increasingly common. Isoniazid resistance, alone and in combination with other drugs, is the commonest pattern of resistance worldwide (Espinal MA *et al.*, 2001). The effect of lone isoniazid resistance on the treatment of pulmonary tuberculosis is minimal provided the drug regimen contains rifampicin (Mitchison DA *et al.*, 1986). Nevertheless, there have been recent reports of treatment failure in isoniazid resistant pulmonary tuberculosis (Coninx R *et al.*, 1999). The impact of isoniazid resistance on the outcome of those with TBM is unknown, but may be more substantial given the uncertain efficacy of rifampicin in the treatment of this disease.

The 'Beijing' genotype of *M.tb*, associated with isoniazid and streptomycin resistance, may have emerged recently in Vietnam (Anh DD *et al.*, 2000). This genotype was discovered in 54% of 563 Vietnamese pulmonary isolates collected in 1998/99, and the association with young age suggested it was responsible for recent transmission and primary infection.

The relationship between age, admission clinical features, HIV infection, drug resistance, genotype, and outcome in those with TBM is uncertain. Furthermore, a comparison of the genotype and drug resistance profiles from CSF and lung *M.tb* isolates collected over the same period has not been performed. The aim of this study was to examine the hypothesis that outcome is dependent upon HIV infection, drug resistance, and bacterial genotype.

7.2 Methods

The adults described in this study were admitted to the HTD Clinical Research Unit as described in **Chapter 2.**

7.2.1 Investigations

Fifty-six cultures containing AFB isolated from the CSF were sent to the Mycobacterial Reference Unit, Dulwich, United Kingdom for identification, drug susceptibility testing, and genotyping. The isolates were identified as *M.tb* by the Accu-probe method (Gen-probe, San Diego, USA), and drug susceptibility testing was performed by the resistance ratio method for isoniazid, rifampicin, pyrazinamide, streptomycin, and ethambutol (Collins CH *et al.*, 1997). In this method the growth of sensitive *M.tb* control strains are compared with a test isolate on a series of media containing different drug concentrations. The 'resistance ratio' of the test isolate is calculated by dividing the minimum inhibitory concentration (MIC) of the test by the modal MIC of the control strains. As doubling dilutions of drug in the media are used the resistance ratio is 1, 2, 4, or 8. Test isolates giving a resistance ratio of 1 or 2 are

reported as susceptible. Those isolates with a resistance ratio of 4 or 8 are reported as resistant and highly resistant respectively.

Genotyping of all *M.tb* isolates was carried out in accordance with a standardised protocol for spoligotyping (Kamerbeek J *et al.*, 1997). Briefly, DNA was prepared from each isolate and subject to PCR analysis using the primers, Dra: 5' –CCG AGA GGG GAC GGA AAC-3', and Drb: 5' –GGT TTT GGG TCT GAC GAC-3'. Chromosomal DNA of *M.tb* H37Rv and *Mycobacterium bovis* BCG were used as positive controls and were included in each PCR and subsequent hybridisation. Sterile double distilled water was the negative control. The PCR products were heat-denatured, and hybridised to the spoligotype membrane (Isogen Biosciences, Asmaarssen, Netherlands). Hybridisation was detected by chemiluminescence (ECL, Amersham, UK) and the patterns analysed for clusters using Microsoft Word software. Patterns were compared against the Beijing and 'Vietnam' spoligotypes described by Anh et al (Anh DD *et al.*, 2000).

7.2.2 Statistical Analysis

Thirty clinical and laboratory variables (**Table 7.1**) from each patient TBM confirmed by culture were recorded in a database. Three questions were addressed using these data:

1. Are there any significant differences between the admission clinical features, drug resistance profiles, genotypes, and outcomes of HIV positive and negative patients?

2. Is resistance to isoniazid and streptomycin, alone or in combination, associated with a worse outcome or any other clinical variable?
3. Are any admission clinical or laboratory features, resistance patterns, or outcome associated with *M.tb* genotype?

Table 7.1

Clinical and laboratory variables included in the analysis

Variables	
Sex	CSF opening pressure
Age	CSF total white cell count
Days of illness before admission	CSF neutrophil %
History of coma	CSF lymphocyte %
Body temperature	CSF/blood glucose ratio
Glasgow coma score on admission	CSF lactate
Cranial nerve palsies	CSF chloride
Hemiplegia on admission	CSF protein
Admission disease stage	CSF ZN stain
Disease stage at start of treatment	Sensitivity to isoniazid
HIV status	Sensitivity to streptomycin
Blood white cell count	*M.tb* spoligotype
Blood neutrophil %	Days of illness to ATC
Blood sodium	Days of in-hospital ATC
Days of admission to ATC	In-hospital survival

Kruskal-Wallis test was used to compare continuous parameters between two or more groups of patients; the chi square test with Yates' correction (or Fisher's exact test) was used for comparison of categorical variables. Variables identified in univariate analysis as associated with the outcome variable (clinical outcome, HIV infection, resistance or *M.tb* genotype) ($p<0.05$) were then incorporated in multivariate logistic regression. Forward stepwise variable selection procedure was used to identify independent predictors. The analysis was performed using 'Statistical Product and Service Solutions' (SPSS) software version 10.0 (Microsoft, USA).

7.3 Results

M.tb was identified in the CSF of 56 adult patients. The mean age was 36 years (15-69 years); 36 (64%) were male. Fifteen adults (27%) presented with Grade I TBM, 19 (34%) with Grade II, and 22 (39%) with Grade III TBM. HIV infection was confirmed according to WHO criteria in 11 (20%) adults: all were male. One patient refused testing but had no risk factors for infection. Twenty-four (43%) died in-hospital: 2 (13%) in Grade I, 7 (37%) in Grade II, and 15 (68%) in Grade III. The mean length of stay in hospital for those who survived was 65 days. **Table 7.2** summarises the clinical data.

Univariate analysis revealed 4 variables that were associated with death: history of pre-admission coma, low admission Glasgow Coma score, admission disease stage, and disease stage at the start of ATC (**Table 7.3**). By multivariate logistic regression analysis,

only admission GCS proved to be independently predictive of in-hospital death (odds ratio 0.73, 95% CI 0.61-0.87, p=0.001).

Table 7.2 Summary of clinical details and drug resistance

Variable	**All patients**	**Isoniazid resistance**	**Streptomycin resistance**	**INH + SM Resistance**
Number (%)	56	9 (16%)	10 (18%)	6 (11%)
Age (years)	33	30	39	37
Median	16-64	15-64	18-64	18-64
range				
Male sex	36 (64%)	7 (78%)	7 (70%)	5 (83%)
n (%)				
MRC grade				
n (%)				
I	15 (27%)	4 (44%)	1 (10%)	1 (17%)
II	19 (34%)	3 (33%)	4 (40%)	3 (50%)
III	22 (39%)	2 (22%)	5 (50%)	2 (33%)
HIV positive	11 (20%)	5 (56%)	6 (60%)	4 (67%)
n (%)				
Mortality	24 (43%)	2 (22%)	6 (60%)	2 (33%)
In hospital				
n (%)				

By univariate analysis five variables were associated with HIV infection: male sex, low peripheral blood white cell count, low CSF total white cell count, isoniazid resistance, and streptomycin resistance (**Table 7.4**). There was no significant difference between the percentage of lymphocytes and neutrophils in the CSF of HIV infected and uninfected adults. Two variables entered the logistic model as independently predictive of HIV infection: a lower CSF total white cell count was weakly associated (odds ratio 0.99, 95% CI 0.98-0.999, p=0.025), whereas streptomycin resistance was strongly associated (odds ratio 43.4, 95% CI 3.67-513, p=0.003).

All isolates were sensitive to rifampicin, pyrazinamide, and ethambutol. Only isoniazid and streptomycin resistance were detected. Nine patients were found to have *M.tb* highly resistant to isoniazid (resistance ratio of 8); 6 of these isolates were also highly resistant to streptomycin. Four adults had *M.tb* highly resistant to streptomycin alone. Streptomycin and isoniazid resistance, either alone or in combination, was not associated with death in hospital. However, those adults with isoniazid resistant *M.tb* were more likely to have HIV infection and streptomycin resistant organisms. Streptomycin resistance was independently associated with HIV infection (odds ratio 7.04, 95% 1.15-45.5, p=0.035) and isoniazid resistance (odds ratio 13.3, 95% CI 2.05-86.5, p=0.007). Sixteen (28%) *M.tb* isolates were of the 'Beijing' spoligotype, 6 (11%) were of the recently designated 'Vietnam' spoligotype (Anh DD *et al.*, 2000), and the rest belonged to a heterogeneous group of 24 unique spoligotypes. There was no significant association

Table 7.3 Admission variables associated with in-hospital mortality by univariate analysis

Variable	Survivors	Died in hospital	P value
Number	32	24	
History of pre-admission coma n (%)	10 (31%)	18 (75%)	0.001
Admission GCS	14	9	0.0002
Median	7-15	4-15	
range			
Admission MRC grade n (%)			
I	13 (14%)	2 (8%)	
II	12 (37%)	7 (29%)	0.003
III	7 (22%)	15 (63%)	
MRC grade at start of ATC n (%)			
I	7 (22%)	1 (4%)	
II	18 (56%)	6 (25%)	0.001
HI	7 (22%)	17 (71%)	

Table 7.4 Admission variables associated with HIV infection by univariate analysis

Variable	HIV positive	HIV negative	P value
Number	11	44	
Male sex n (%)	11 (100%)	24 (55%)	0.004
Total White cells in blood	7200	9800	0.023
(10^3/ml) Median range	2660-13800	5199-16500	
Total white blood cell count in	152	356	0.007
CSF (10^3/ml) Median range	34-430	100-1435	
Resistance to isoniazid n (%)	5 (45%)	4 (9%)	0.011
Resistance to streptomycin n (%)	6 (55%)	4 (9%)	0.002

Table 7.5 Clinical variables and *M.tb* spoligotype

Variable	Beijing spoligotype	Vietnam spoligotype	Other spoligotype	P value
Number	16 (28%)	6 (11%)	34 (61%)	
Age (years) median range	33 16-50	51 26-67	31 15-64	0.060
Male Sex n (%)	12 (75%)	3 (50%)	21 (62%)	0.490
HIV infection n (%)	3 (20%)	1 (17%)	7 (21%)	1.000
Isoniazid resistant n (%)	3 (19%)	0 (0%)	6 (18%)	0.749
Streptomycin resistant n (%)	5 (31%)	1 (17%)	4 (12%)	0.176
Combined INH and SM resistance n (%)	3 (19%)	0 (0%)	3 (9%)	0.422
Mortality in hospital n (%)	7 (44%)	3 (50%)	14 (41%)	1.000

between the 'Beijing' genotype and HIV infection, younger age, drug resistance, or death (**Table 7.5**).

Nor does isoniazid resistance appear to slow clinical recovery: adults with resistant organisms were discharged from hospital after a median of 47 days (range 11-105 days), whereas those with sensitive organisms left hospital after a median of 71 days (range 25-153 days).

7.4 Discussion

This study explores the relationship between HIV infection, drug resistance, *M.tb* genotype, and the outcome of adults with TBM. The insensitivity of CSF culture for the diagnosis of TBM has limited study of these areas, as an organism is not isolated in many treated cases. Univariate and multivariate analyses of the variables predicting death in this series showed that the level consciousness, assessed by Glasgow coma score, predicted death most reliably. This is consistent with previous studies (Hosoglu S *et al.*, 2002; Humphries MJ *et al.*, 1990; Kalita J *et al.*, 1999), and emphasises the importance of early diagnosis and treatment. However, the relationship between the duration of pre-admission symptoms and prognosis may not be linear. Nine adults in this series died despite presenting with less than 10 days of symptoms, compared with 15/42 presenting with more than 10 days of symptoms (p=0.06). The rate of disease progression is unpredictable. There may be adults with rapidly progressive TBM who would benefit particularly from early treatment and this merits further research.

Previous reports suggest that HIV infection does not alter the clinical features and in-hospital mortality of TBM (Berenguer J *et al.*, 1992; Dube MP *et al.*, 1992). In this study, peripheral blood and CSF white cell counts were significantly lower in HIV infected adults. This finding has been reported previously in CSF (Dube MP *et al.*, 1992), but not blood and may create clinical diagnostic difficulties. TBM with few white cells in the CSF can occur in the HIV infected and the elderly (Karstaedt AS *et al.*, 1998), and the diagnosis should always be suspected in these groups despite atypical laboratory findings. All of the HIV infected patients in this study were male: the majority (8/11) admitted intra-venous drug use, a male dominated habit in Ho Chi Minh City. The in-hospital mortality of the HIV infected and uninfected adults with TBM were not significantly different. A larger cohort, with more prolonged follow-up is required to confirm this preliminary finding.

HIV infected adults with TBM were more likely to be infected with *M.tb* resistant to isoniazid (p=0.011) and streptomycin (p=0.002). Streptomycin resistance was independently predictive of HIV infection, supporting the local policy for treating HIV infected adults with ethambutol rather than streptomycin. Why those with HIV should be infected with more resistant organisms is unclear. Common behavioural patterns may result in the spread of resistant organisms amongst the intra-venous drug users in the city, although the heterogeneous spoligotype patterns of the *M.tb* isolates from these patients do not support this suggestion. Alternatively, as HIV infection probably shortens the time between primary *M.tb* infection and the onset of TBM, the CSF *M.tb* isolates from these

adults may show similar resistance profiles to contemporary pulmonary isolates. The similar incidence of isoniazid resistant [119/499 (24%)] and streptomycin resistant [156/499 (31%)] pulmonary isolates from Ho Chi Minh City in 1998/99 supports this suggestion (Anh DD *et al.*, 2000).

Resistance to isoniazid or streptomycin, or both, was not associated with increased in-hospital mortality. Of adults with *M.tb* sensitive to all 4 drugs 42% (18/43) died, compared to 22% (2/9) with an isolate highly resistant to isoniazid. Death from TBM usually occurs within the first month of treatment (Girgis NI *et al.*, 1998). In this study only 2/24 (4%) deaths occurred after 28 days of ATC; both these patients were infected with fully sensitive organisms.

Although this study has limited power, and there are no data available on long-term outcome measures, these results suggest isolated isoniazid resistance has little prognostic impact in adults with TBM. This is a surprising finding given the important role in the treatment of TBM attributed to isoniazid and there are a number of possible explanations. First, the CSF levels of isoniazid may be sufficiently high to have some bactericidal effect despite in vitro resistance. Ellard et al showed that 4 hours after an oral dose of about 9mg/kg isoniazid the mean CSF concentration was 3.2mg/l; over 30 times the MIC of isoniazid against sensitive *M.tb* (Ellard GA *et al.*, 1993). Assuming dose-linearity, the CSF levels achieved using 5mg/kg isoniazid, as in this study, would be expected to be approximately 16 times higher than the MIC of sensitive isolates and twice that of 'highly resistant' isolates. Second, the action of the other anti-tuberculosis drugs may be

sufficient to negate the effect of isoniazid resistance. This would place fresh emphasis on the role of rifampicin in the treatment of TBM, and suggests it may be of equal importance to isoniazid. Third, isoniazid resistant organisms may be less virulent. There are data that suggest isoniazid resistant strains of *M.tb* are less virulent than drug-susceptible isolates in guinea pigs; although more recent studies suggest there is no significant loss of bacterial fitness resulting from mutations in the *KatG* gene responsible for conferring resistance to isoniazid in more than half of *M.tb* clinical isolates (Pym AS *et al.*, 2002) (see also **Table 1.1**).

Definite conclusions cannot be drawn without more detailed modelling of the pharmocokinetic and pharmacodynamic properties of the anti-tuberculosis drugs in TBM. However, these results suggest that isoniazid resistance, with or without streptomycin resistance, does not affect the outcome of TBM as long as four drugs are used. These findings also suggest that doses of isoniazid greater than 5 mg/kg may not improve outcome in the treatment of TBM, provided rifampicin is part of the regimen.

Of the 56 *M.tb* isolates 28% (16/56) were of the 'Beijing' genotype. This was not associated significantly with age, admission clinical features, HIV status, drug resistance, or outcome. Over the same period Anh et al reported that 53% of pulmonary isolates from Ho Chi Minh City were the 'Beijing' genotype (Anh DD *et al.*, 2000), significantly more than recovered from CSF ($p<0.001$). Resistance profiles from lung and CSF isolates were not significantly different. A number of hypotheses may explain the smaller proportion of 'Beijing' genotype found in the CSF. Firstly, *M.tb* responsible for

meningitis in adults may have been acquired at a time when the 'Beijing' genotype was less prevalent in Vietnam. However, the 'Beijing' genotype in CSF was not associated with younger age, or with HIV infection. Secondly, the 'Beijing' genotype may be less neurovirulent than other strains. Genotype-dependent neurovirulence may occur (Arvanitakis Z *et al.*, 1998), although a predominant genotype causing TBM was not found in this study. Thirdly, the Vietnamese immune response to 'Beijing' *M.tb* may reduce the likelihood of dissemination to the central nervous system. Further study is required to address these hypotheses.

In conclusion, this study confirms the importance of diagnosing and treating TBM before the onset of coma. In Vietnam, HIV infection is associated with isoniazid and streptomycin resistant organisms, but isoniazid resistant *M.tb* is not associated with increased in-hospital mortality. This finding suggests three possible explanations: the CSF levels of isoniazid may still have significant activity against 'highly resistant' organisms; rifampicin and the other 2 drugs are more active than previously thought; or *M.tb* resistant to isoniazid causes less severe disease. This study supports the use of ethambutol over streptomycin in HIV infected adults in Vietnam, but does not support the use of higher dose isoniazid in combination with rifampicin for the routine management of adults with TBM.

CHAPTER 8

DISCUSSION

The aim of this thesis was to address three important questions:

1. What is the best method for distinguishing TBM from other central nervous system disorders?
2. How does disease pathophysiology relate to treatment and clinical outcome?
3. What other variables influence outcome?

The extent to which these questions have been answered will now be discussed.

8.1 The diagnosis of tuberculous meningitis

The diagnosis of TBM has exercised physicians ever since it became a treatable disease in 1948; and the significant association between early diagnosis, treatment and survival accentuates its importance. There are three major impediments to diagnosis. First, the presenting clinical features of the disease are non-specific. Second, low concentrations of bacilli in the CSF reduce the sensitivity of conventional bacteriology. Third, alternative diagnostic methods have not been properly assessed. These problems are increased in settings with limited resources, where most cases of TBM are seen. Therefore, the answer to the first question posed by this thesis may be different depending upon where it is asked.

Chapter 3 examines whether TBM can be distinguished from bacterial meningitis without the help of a microbiology laboratory. The results suggest it can: prospective assessment of a diagnostic rule based upon the values of 5 clinical features demonstrated 86% sensitivity, and 79% specificity.However, the development and use of these algorithms are greatly affected by tuberculosis and HIV prevalence. Co-infection with HIV may alter the presenting features of TBM as reported in **Chapter 7**, and changes the spectrum of disorders presenting with similar clinical syndromes. In particular, the algorithms were not developed to distinguish TBM from cryptococcal meningitis, a common cause of meningitis in HIV-infected individuals. Alternative algorithms need to be developed and tested in areas with high prevalence of HIV infection.

The value of conventional bacteriology for the diagnosis of TBM is often debated. Old reports suggested AFB could be found in nearly every case if the microscopist was prepared to look hard (Stewart SM, 1953), but this has rarely been the experience of contemporary laboratories (Garg RK, 1999). Only Kennedy, in more recent times, was able to demonstrate that repeated CSF sampling improved the sensitivity of ZN stain to over 80% (Kennedy DH *et al.*, 1979). The factors that determine the sensitivity of bacteriology have not been previously examined systematically: **Chapter 4** confirms that it is possible to make a bacteriological diagnosis of TBM in more than 80% of patients, and demonstrates the volume of CSF examined is independently predictive of either seeing or culturing *M.tb* from the CSF. These are critical data when trying to assess the

best diagnostic test for TBM and suggest simple methods can dramatically improve the performance of conventional bacteriology.

The value of commercial molecular diagnostic tests for TBM is unclear. Previous attempts to clarify the diagnostic role of NAA have failed because of low numbers of cases and inadequate bacteriological diagnostic comparison. In theory, molecular methods should improve upon bacteriology, but the data presented in **Chapter 5** and the results of a recent meta-analysis suggest otherwise (Pai M *et al.*, 2003). These authors calculate that the sensitivity from 14 studies (110 CSF specimens from patients with TBM) with commercial NAA was 56% (95% CI 46-66%), and conclude that their overall low sensitivity precludes the use of these tests to rule out TBM with certainty. The results in this thesis from 79 adults with TBM strengthen this conclusion: the sensitivity of MTD before the start of treatment was 38% (95% CI 26-51%). If the results of previous studies using MTD are combined with this result (see **Table 5.1**), the overall sensitivity and specificity of the test is 50% (69/138) (95% CI 41-58%) and 99% (569/575) (95% CI 98-100%). These data strongly suggest that careful bacteriology is as good, or better, than the MTD. However, a combination of ZN stain and MTD on serial samples detected 83% of cases (95% CI 71-92%); and after treatment has started the MTD test retained sensitivity for longer than ZN stain or culture, presumably reflecting continued detection of rRNA from non-viable organisms.

In conclusion, in settings without microbiological facilities a diagnostic algorithm based on simple clinical features will help distinguish TBM from other severe central nervous

infections. In all other settings the meticulous microscopy of large volumes of CSF remains the best single diagnostic test. A combination of careful bacteriology and MTD may help in the rapid detection of over 80% of cases. However, the fatal consequences of delayed treatment demand a more sensitive single diagnostic test. Alternative solutions will be discussed later.

8.2 The pathophysiology of tuberculous meningitis

The second part of this thesis examined the pathophysiology of TBM before and after starting treatment and defined the variables that influence outcome. The purpose of this section is to bring together the findings and suggest a coherent scheme of events that lead to death or survival.

The pathogenesis of TBM begins with a bacteraemia secondary to a pulmonary infection. Primary progressive pulmonary tuberculosis and HIV infection increase both the duration of bacteraemia (Bouza E *et al.*, 1993) and the likelihood of disseminated tuberculosis (Iseman MD, 2000). Differences in host genetic susceptibility and mycobacterial virulence may also govern the probability of dissemination, although these factors have not been addressed in this thesis. The clinical onset of the TBM begins with the release of *M.tb* into the subarachnoid space from a Rich focus (Rich AR *et al.*, 1933). Evidence presented in **Chapters 4** and **6** suggest that outcome after this event is dependent upon the relationship between bacillary load and the host immune response and is best predicted by CSF lactate concentration.

Chapter 4 demonstrates that the likelihood of seeing or culturing *M.tb* from the CSF before treatment was independently associated with larger volumes of CSF examined, longer duration of preceding symptoms, higher CSF neutrophil counts, lower CSF:blood glucose ratios and higher CSF lactate concentrations. **Chapter 6** reveals that death was associated with three of these five variables in a sub-section of the same population: CSF neutrophil count, glucose ratio, and lactate. The concentrations of CSF lactate and glucose are inversely related to one another and their concentrations reflect the degree of tissue hypoxia and anaerobic glycolysis (Fishman R, 1992). As such, they are the consequence not the cause of the cerebral pathology. The role of neutrophils in the CSF appears more complex: although higher numbers were indirectly associated with more bacilli in the CSF, they were also associated with increased survival. These data suggest they may have an important role in a successful inflammatory response to cerebral *M.tb*, which is supported by the electron micrographs showing bacilli within the phagasomes of neutrophils. Greater proportions of CSF neutrophils have been described in early disease (Garg RK, 1999), which could account for the association with better outcome, but there was no relationship between duration of symptoms and CSF neutrophil counts in these studies.

The cellular CSF response to *M.tb* infection is coordinated by cytokines and chemokines (Flynn JL *et al.*, 2001). The host response to increasing numbers of cerebral bacilli appears highly compartmentalised, characterised by increased CSF concentrations of IL-8, TNF-α, and IFN-γ, with little or none of these molecules detectable in paired plasma.

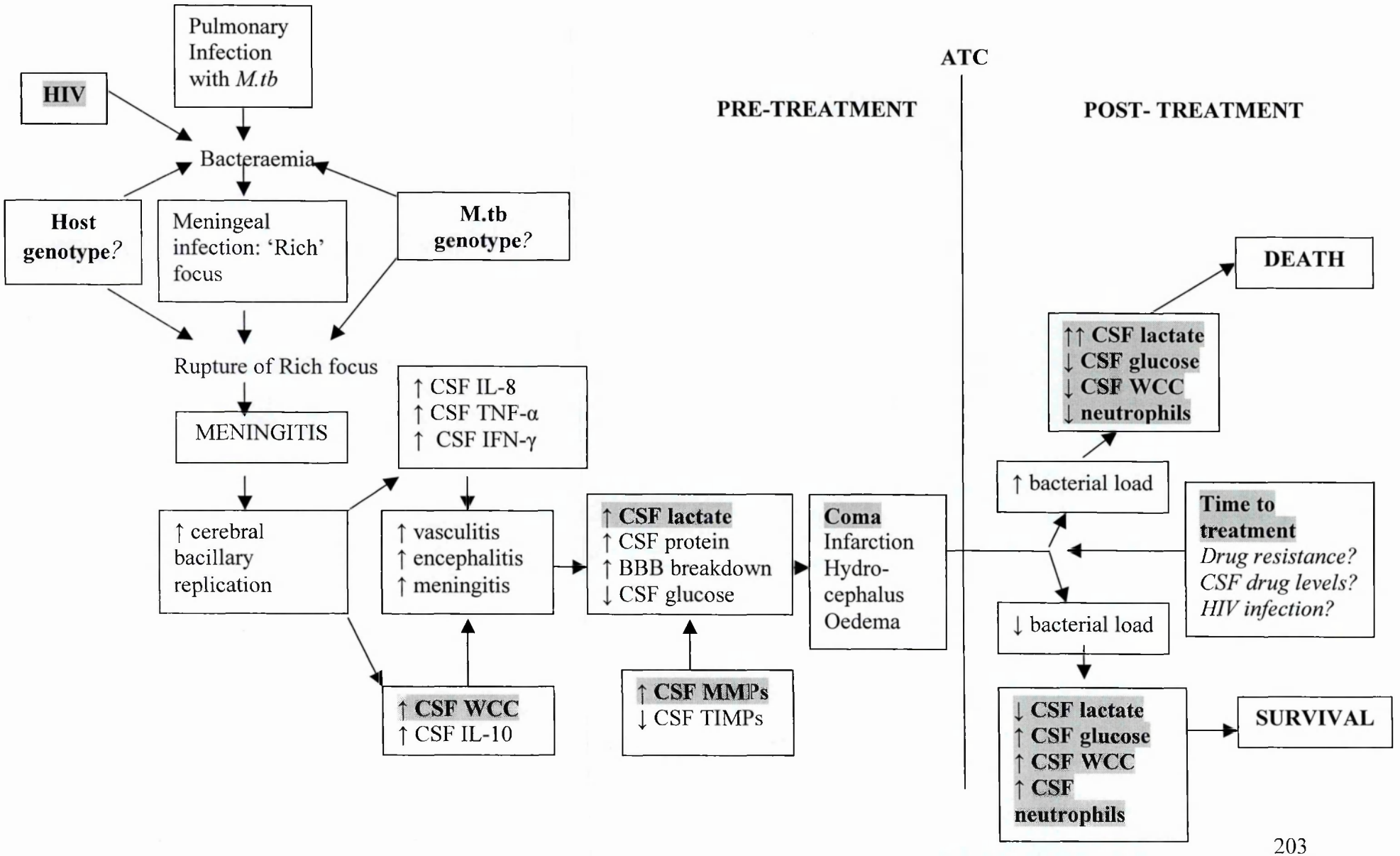
Although IL-8, TNF-α, and IFN-γ did not correlate directly with outcome, they are all significantly correlated with CSF lactate concentration, which suggests they may have a concentration dependent effect upon immune activation and cerebral inflammation. However, there was no correlation between CSF concentration of IL-8 (a potent neutrophil chemo-attractant) and CSF neutrophil numbers. Only CSF IL-10 concentrations were significantly correlated with CSF cellular response.

The conclusions drawn from **Chapter 6** are limited. CSF may not accurately reflect disease at the site of infection, or the concentrations of cytokines their biological activity. These types of studies would be improved by attempting to correlate changes in CSF inflammatory molecules with serial MRI documenting macroscopic brain parenchymal changes such as infarction, tuberculoma formation, and hydrocephalus. **Figure 8.1** presents an overview of the pathogenesis of TBM and highlights variables of proven and speculative prognostic importance addressed in this thesis. Unfortunately, these and previous studies do not suggest novel rational interventions aimed at modifying critical aspects of disease pathogenesis.

8.3 Other variables that influence prognosis

Early treatment with standard anti-tuberculosis drugs remains the largest contribution a physician can make to improve outcome. The severity of coma on admission proved to be independently predictive of death (OR 0.73, 95% CI 0.61-0.87, p=0.001), although the duration of illness before admission did not correlate with coma score or outcome.

Figure 8.1. Overview of the pathophysiology of TBM with confirmed (shaded) **and possible prognostic variables**



This may be because the relatives of comatose patients (on whom the physicians must rely for the history) underestimate the length of symptoms. Alternatively, there may be more virulent strains of *M.tb* that cause more rapidly progressive disease; or some people may be more susceptible to rapid disease progression than others. **Chapter 7** suggests the first explanation is less likely as no relationship was observed between spoligotype and duration of symptoms and outcome. However, the numbers of isolates in this study was small, and evidence is accumulating that suggest different strains of *M.tb* are capable of producing different clinical phenotypes (Manabe YC *et al.*, 2003). Future studies aimed at investigating this possibility will be discussed later.

The influence of host susceptibility on TBM progression and outcome is poorly defined, even with respect to co-infection with HIV. **Chapter 7** reports that adults with HIV and TBM presented with lower numbers of white cells in their blood and CSF and were more likely to be infected with *M.tb* resistant to streptomycin and/or isoniazid. These findings have implications for diagnosis and treatment: low or even absent white cells in the CSF may mask the diagnosis of TBM in HIV infected individuals, and the likelihood of resistant organisms suggest alternative drugs should be considered in this population. However, HIV infection and/or isoniazid/streptomycin resistance did not alter in-hospital mortality. This is surprising, particularly when data in **Chapter 4** suggests adults with HIV have greater numbers of CSF bacilli, and **Chapter 5** shows resistance to isoniazid/streptomycin slows bacterial clearance from the CSF. These findings must be

confirmed by larger studies with longer follow-up (at least 9 months from start of treatment).

8.4 Future directions

8.4.1 Diagnosis: alternative strategies

This thesis shows novel approaches to the diagnosis of TBM are still required, although their development cannot be divorced from the settings in which they are most needed i.e. the developing world. Suitable tests must be cheap, easy to perform and must have reagents with long shelf lives that do not require refrigeration. However, these specifications should not hinder research and development of new assays: commercial kits can simplify techniques, costs may reduce and laboratories may improve. The most potent reason for few new diagnostic tests for TBM is their lack of commercial possibility: the populations that need them most can afford them least.

But this may change. Increased global concern regarding pulmonary tuberculosis has lead to the development of several new diagnostic assays, which could be adapted for use on CSF. The identification of immunodominant antigens such as ESAT-6 and antigen 85A/B have suggested novel diagnostic assays for the detection of tuberculosis infection. An enzyme-linked immunospot (ELISPOT) assay for IFN-γ identified ESAT-6-specific T cells in the blood as an accurate marker of *M.tb* infection (Lalvani A *et al.*, 2001). A commercial whole-blood assay has been developed to detect IFN-γ production to this and other antigens that may be more appropriate for use in resource-limited settings

(Johnson PD *et al.*, 1999). It is not known whether either of these methods could be successfully adapted for use on CSF. The low numbers of lymphocytes in the CSF would necessitate taking large (>5mls) volumes of CSF to obtain sufficient cells, which would be impossible in some cases (particularly children) and reduce the likelihood of bacteriological diagnostic confirmation in most others. It is also unclear whether these assays can distinguish between past infection and current disease, which could be a problem in areas of high tuberculosis prevalence.

The detection of various *M.tb* antigens in the CSF by ELISA has a short history that deserves revisiting (Mathai A *et al.*, 1994; Radhakrishnan VV *et al.*, 1990; Sada E *et al.*, 1983; Watt G *et al.*, 1988). New possibilities stem from the complete sequencing of the *M.tb* genome (Cole ST *et al.*, 1998), which offers a unique opportunity to identify novel diagnostic targets. The availability of this sequence enables screening of complete protein families that contain immunodominant molecules, such as ESAT-6 (Louise R *et al.*, 2001). Subcellular protein fractions of *M.tb* can be resolved by two-dimensional liquid phase electrophoresis, and particular fractions isolated that stimulate dominant T cell responses. Individual proteins can be identified within these fractions and their DNA sequence predicted (Covert BA *et al.*, 2001). This 'post-genomic' approach has not yet been employed upon the CSF from patients with TBM, and may identify novel detectable antigens. In addition, the growing availability of monoclonal antibodies to a range of *M.tb* antigens and peptides will improve the specificity of these assays.

Immunodominant antigens, identified by the methods described above, may not be secreted in the CSF in sufficient concentrations to enable consistent detection by ELISA, and other molecules and methods of detection should be considered. There are inconclusive reports suggesting that tuberculosteric acid, a component of *M.tb* cell wall, can be detected by gas-liquid chromatography of CSF from patients with TBM (Brooks JB *et al.*, 1990). Advances in analytic techniques, for example liquid chromatography – mass spectroscopy, suggest these methods may now be capable of identifying and consistently detecting molecules unique to the CSF of those with TBM at pico-molar concentrations. Although these methods are a long way from incorporation into the conventional diagnostic laboratory, they deserve further investigation.

Recently, bacteriophages have been used to identify *M.tb* in sputum, and to assess drug susceptibility (Banaiee N *et al.*, 2001; Banaiee N *et al.*, 2003; Park DJ *et al.*, 2003; Wilson SM *et al.*, 1997). Two methods are described. The first uses a phage carrying the firefly luciferase gene that infects viable *M.tb* labelling it with the ability to produce light (Banaiee N *et al.*, 2001). The second uses a phage that infects viable *M.tb*, and can be transferred to infect a plate of fast-growing mycobacteria causing visible areas of lysis (Wilson SM *et al.*, 1997). The considerable advantage of the later method is that it does not require special laboratory equipment and may be better suited to resource-poor settings. Field trials have suggested that this assay performs well in specimens other than sputum (Marei AM *et al.*, 2003), although there are no reports of its use on CSF.

It remains to be seen whether this assay has sufficient sensitivity to detect the small numbers of bacilli found in the CSF.

8.4.2 Pathophysiology and outcome: what can we improve?

Improving outcome from TBM remains a major priority and one that has yet to be properly addressed. Prevailing dogma suggests that poor prognosis results from an inappropriate immune response within the confined space of the brain. As discussed earlier, the evidence to support this view is poor. Animal models have suggested an important role for TNF-α (Tsenova L *et al.*, 1999), and demonstrated improved outcome following TNF-α inhibition by thalidomide (Tsenova L *et al.*, 1998). However, like previous reports, this thesis was unable to confirm a relationship between CSF TNF-α concentration and outcome in humans, although concentration in the CSF may have little relation to biological activity at the site of disease. Use of adjunctive thalidomide (in addition to corticosteroids) in humans has been reported with inconclusive results (Schoeman JF *et al.*, 2000; Roberts MT *et al.*, 2003) and has yet to be subject to a controlled trial.

Despite poor understanding of the pathogenesis of TBM, physicians continue to advocate adjuvant corticosteroids in the face of meagre data from clinical trials. Putative mechanisms of action of corticosteroids in this setting include down regulation of T-cell functioning; regulation of a range of cytokines; and impairing the blood-brain barrier dysfunction via interference with cell adhesion, migration, and matrix metalloproteinase

activity. A large, randomised controlled trial is urgently required, in parallel with studies assessing the effect of corticosteroid upon CSF cellular phenotypes (by flow cytometry), cytokine expression and blood-brain barrier integrity. If an adequately powered clinical trial demonstrates an impact upon outcome, CSF studies might clarify how corticosteroids alter pathogenesis and identify new molecules of prognostic importance. These studies are currently being performed at HTD, but further investigations could consider techniques employed by those investigating the pathogenesis of multiple sclerosis, a common inflammatory disease of the developed world. In particular, cDNA micro arrays have been employed for gene-expression analysis of peripheral blood mononuclear cells from patients treated with different immunomodulatory drugs. Using these methods, several genes involved in innate and specific immune responses have been identified that are potential effector targets for the drugs on trial for this disease (predominantly interferon beta) (Wandinger KP *et al.*, 2001; Wandinger KP *et al.*, 2003). A similar approach could be adopted with TBM, exploring potentially important genes in pathogenesis by using host cDNA micro arrays of mononuclear cells from blood and CSF taken over the course of treatment with corticosteroids, or other candidate drugs such as thalidomide.

Imaging techniques promise complementary insights into disease pathogenesis, in particular on the development and response to treatment of infarctions, tuberculomas, and hydrocephalus. MRI is increasingly available, and serial scans on consecutive patients would offer much valuable data regarding the timing, typical anatomical locations, and

resolution of these complications. Doppler ultra-sound studies have been used to assess the nature and extent of TBM-related cerebral vasculopathy (Kilic T *et al.*, 2002), but magnetic resonance angiography (MRA) would provide greater resolution and allow arterial changes to be studied in anatomically defined areas. The techniques of functional MRI and single photon emission computorised tomography (SPECT) may provide further data regarding regional cerebral blood flow, perfusion and parenchymal metabolic activity (Kalita J *et al.*, 2002). These techniques could provide a powerful way of investigating TBM pathogenesis and assessing response to novel interventions, particularly if used in conjunction with the molecular and cellular investigations described above.

Future research should also be directed at investigating why some patients infected with *M.tb* develop meningitis, and others do not. There may be genetic and environmental reasons why some patients are more susceptible to this form of the disease (Bloom BR *et al.*, 1998). Alternatively, there may be strains of *M.tb* that are more likely to cause cerebral infection, and this possibility is explored in **Chapter 7**. There is however, very little information on the molecular characteristics of *M.tb* strains associated with different clinical phenotypes or outcomes. Although large numbers of isolates would be required, it may be possible to compare strains of *M.tb* from the CSF and lung by RFLP, spoligotyping or mycobacterial interspersed repeat units (MIRU) typing (Barnes PF *et al.*, 2003), and identify certain strains more commonly isolated from the CSF or the lung. The biological characteristics of these organisms could be further characterised by

comparing disease phenotype in animal models, infection characteristics in macrophage/monocyte cell lines, and *M.tb* gene expression in different environments by micro array.

The outcome of future patients with TBM is threatened by increasing *M.tb* resistance to the first-line anti-tuberculosis drugs. Although the impact of isoniazid and/or streptomycin on outcome is uncertain, the treatment of multi-drug resistant TBM is severely impaired by the high likelihood of death before the results of susceptibility tests are known (Daikos GL *et al.*, 2003). Two areas require urgent further investigation: the rapid prediction or detection of resistant *M.tb* in the CSF, and the CSF pharmacokinetcs and pharmacodynamics of second line drugs.

Conventional drug susceptibility testing takes too long to be clinically relevant for TBM, therefore alternative methods need to be considered. It may be possible to develop clinical algorithms to predict the likelihood of drug resistant *M.tb* based upon admission data, CSF parameters, and clinical response over the first 3-5 days of ATC. These algorithms may be good at assessing the chance of resistance in a given community, but are unlikely to have sufficient sensitivity and specificity to be used alone and in different populations. NAA assays have been developed that detect mutations in the *M.tb* rpoB gene that predict phenotypic rifampicin resistance (Mokrousov I *et al.*, 2003). Lone rifampcin is rare in most settings and is usually found in combination with isoniazid resistance. Therefore, this assay has been shown to be useful in the early prediction of multi-drug resistant pulmonary disease (Johansen IS *et al.*, 2003). The sensitivity and

specificity of the assay for the diagnosis of rifampicin resistant TBM is unknown, and requires investigation. Phage based systems for rapid drug susceptibility testing, as described above, are unlikely to be useful as they currently require mycobacterial populations that may take 1-2 weeks to cultivate from CSF.

Finally, the treatment of drug resistant tuberculosis presents an enormous challenge to future researchers. It is not clear which drugs constitute the best second line drug regimen and there are no published controlled trials addressing this issue for any form of tuberculosis (Frieden TR *et al.*, 2003). The treatment of drug resistant TBM is made especially difficult, as any potential drug must also penetrate the BBB. Fluoroquinolones are bactericidal for *M.tb* (Berning SE, 2001) and WHO recommends them for the treatment of multi-drug resistant pulmonary tuberculosis (Crofton J *et al.*, 1997), but their use in TBM is restricted to case reports (Berning SE *et al.*, 2001). Data regarding CSF penetration and pharmacokinetics are scant (Fish DN *et al.*, 1997; Nau R *et al.*, 1990) and it is uncertain which fluoroquinolone represents the best drug for treating TBM. The CSF pharmacokinetics and pharmacodynamics of ciprofloxacin, levofloxacin, gatifloxacin and moxifloxacin requires investigation before randomised controlled trials can be proposed. Limitations on the frequency of lumbar punctures suggest these studies should employ population pharmacokinetic designs and analysis. These techniques allow the variability in drug concentration or pharmacological effect to be studied between individuals when standard dosage regimens are administered, and are especially suited to the type of sparse data TBM CSF studies may produce (Sun H *et al.*, 1999).

8.5 Concluding comments

The outcome from TBM has not improved since the introduction of isoniazid in 1952, and neither have diagnostic methods. This thesis suggests simple clinical methods are useful, but on its 120th anniversary, the Ziehl-Neelsen stain remains the best available single diagnostic test for TBM. Laboratories with the most money and time should consider the combined performance of meticulous bacteriology and MTD on repeated samples, as this approach detected the most cases. Laboratories with fewer resources could identify patients who most warrant this approach by clinical diagnostic algorithms. This thesis has shown that treatment early in the disease before the onset of coma is the best way to prevent death, but the pathogenesis of TBM remains elusive. CSF lactate is an important CSF marker of disease severity and bacterial load, and neutrophils may have an important and hitherto unreported protective role, but the cellular and molecular response to infection requires greater characterisation.

Further research is urgently required to evaluate the diagnostic role of new antigen and phage-based assays; to confirm or refute the value of adjunctive corticosteroids; to identify new drugs for the treatment of resistant *M.tb*; and to identify the mechanisms of disease that may be amenable to novel interventions. If these problems are not addressed the outcome of patients admitted to the Hospital for Tropical Diseases in Ho Chi Minh City and elsewhere will not improve for another 50 years.

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APPENDIX: PUBLICATIONS ARISING FROM THIS THESIS

Thwaites G, Chau TT, Mai NT, Drobniewski F, McAdam K and Farrar J. **2000.** Tuberculous meningitis. *J Neurol Neurosurg Psychiatry,* 68, 289-99

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Thwaites GE, Caws M, Chau TT, Dung NT, Campbell JI, Phu NH, Hien TT, White NJ and Farrar JJ. Conventional bacteriology compared with nucleic acid amplification (the Amplified Mycobacterium Direct Test) for the diagnosis of tuberculous meningitis before and after starting anti-tuberculosis chemotherapy. *J Clin Microbiol*

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